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(57) Abstract

An efficient and versatile method of forming N-linked glycoconjugates is described wherein a glycosyl acceptor, typically comprising an activated carboxyl group, is reacted with a glycosylating agent, typically a glycosylamine, in the presence of a coupling catalyst and optionally an exogenous base. Depending on the choice of reactive site, this method can be utilized to form N-linked glycoconjugates, in either a soluble or substrate-bound, linear or branched format.

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METHODS FOR THE SOLID PHASE SYNTHESIS OF GLYCOCONJUGATES

CROSS-REFERENCE

This application is a continuation-in-part of copending U.S. patent application Serial No. 08/201,607, filed February 25 1994, which is a continuation-in-part of copending U.S. patent application Serial No. 08/179,741, filed January 11, 1994. Both of these applications are expressly incorporated herein by reference for all purposes.

BACKGROUND OF THE INVENTION

The present invention is related to the area of carbohydrate and polymer synthesis, and, more specifically, to methods for producing N-linked glycoconjugates. The invention has application in the fields of medicinal and polymer chemistry and pharmacology.

Glycoamino acids and glycopeptides (or glycoproteins) comprise one of the most important contemporary areas in chemistry and biology. Much of the recent interest in this area stems from the realization that glycosylation can significantly alter the therapeutic profile, (*see* Cumming (1991) Glycobiology 1:115 and Gribben *et al.* (1990) Lancet 434) through significantly influencing important biological and physiochemical parameters, of protein pharmaceuticals such as tPA (*see* Parekh *et al.* (1989) Biochemistry 28:7644; Witter *et al.* (1989) Biochemistry 28:7662 and Parekh *et al.* (1989) Biochemistry 28:7670) and EPO (*see* Narhi *et al.* (1991) J. Biol. Chem. 266:23022; Dordal *et al* (1985) Nature (London) 116:2293; and Dube *et al.* (1988) J. Biol. Chem. 263:17516). For example, pharmacologically important variables that can be affected by glycosylation include circulatory life-time (*see* Takuechi *et al.* (1989) Proc. Natl. Acad. Sci. USA 86: 719; Galili *et al.* (1989) Proc. Natl. Acad. Sci. USA 86:719; and Cebon *et al.* (1990) J. Biol. Chem. 265:3671); solubility (*see* Middagh (1987) J. Biol. Chem. 262:4483); immunogenicity (*see* Alexander and Elder (1984) Science

226:1328); biodistribution (*see* Ashwell and Harford (1982) Ann. Rev. Biochem. 51:531); and proteolytic stability (*see* Olden *et al.* (1982) Biochim. Biophys. Acta 651:209).

In addition, glycosylation can play a significant role in protein folding (*see* Smith and Pease (1980) CRC Crit. Rev. Biochem. 8:315); protein transport (*see* Sando and Nuefeld (1977) Cell 12:619 and Kaplan *et al.* (1977) Proc. Natl. Acad. Sci. USA 74:2026); and numerous recognition phenomena (*see, e.g.,* Race and Sanger in *Blood Groups in Man,* 6th ed., Blackwell Scientific, Oxford (1975); Baeckstrom *et al.* (1991) J. Biol. Chem. 266:21537; and Sharon and Lis (1989) Science 246:27). Complex glycosides attached to exterior cell surfaces (N-linked glycopeptides, O-linked glycopeptides, and glycolipids) are involved in the regulation of cell metabolism, host-pathogen interactions, tumor cell metastasis, cell-cell recognition, cell adhesion, and cell development. *See* Montreuil (1980) Adv. Carbohydr. Chem. Biochem. 37:157-223.

Glycopeptides consist of oligosaccharide units attached to a polyamide core via glycosidic bonds. The oligosaccharide chains vary in size from one to thirty or more units. Only one or a few carbohydrate units are found in some proteins, while in others up to eight hundred sugar side chains have been identified. *See* Kobata and Ginsburt (1984) Biology of Carbohydrates Wiley: New York. Although over a hundred monosaccharides are known in nature, only a small number have been identified in naturally-occurring glycopeptides. *See* Kornfeld and Kornfeld (1976) Ann. Rev. Biochem. 56:497.

The vast majority of glycosidic bonds between sugar and protein are either N-linked or O-linked. In naturally occurring N-linked glycopeptides, typically a β-N-glycosidic linkage is made between an N-acetyl glucosamine residue to the side chain amide function of asparagine. *See* Johansen *et al.* (1961) Biochem. J. 78:518. However, recently several exceptions have been reported in the literature. The presence of N-glucosyl and N-galactosyl aminylasparaginyl units in some cell surface glycopeptides isolated from halobacteria has been detected (*see* Wieland *et al.* (1983) Proc. Natl. Acad. Sci. USA 80:5470 and Paul *et al.* (1986) J. Biol. Chem. 261:1020), as has the existence of an α-N-glycosidic bond between glucose and glutamine in a nephitogenic glycopeptide (*see* Shibata *et al.* (1980) Carbohydr. Res. 86:316 and Takeda *et al.* (1985) Carbohydr. Res. 139:133).

The synthesis of glycoamino acids and glycopeptides is a relatively new area of study. *See* Micheel and Kochling (1958) Chem. Ber. 91:673. A number of N-glycoamino acids have been constructed by condensing an glycosyl amine with an appropriately protected amino acid in the presence of DCC. *See* Micheel and Kochling (1960) Chem. Ber. 93:2372; Garg and Jeanloz (1976) Carbohydr. Res. 49:482; Jacquinet *et al.* (1974) Carbohydr. Res. 32:482; Marks and Nueberger (1961)J. Chem. Soc. 83:4872); Marshall and Nueberger (1964) Biochemistry 3:1596; Yoshimura and Hashimoto (1967) Carbohydr. Res 4:435; Yoshimura *et al.* (1967) Carbohydr. Res. 5:82; Otvos *et al.* (1989) Pept. Res. 2:362-366; Anisfield and Lansbury (1990) J. Org. Chem. 55:5560-5562; and Kiyozumi *et al.* (1970) Carbohydr. Res. 14:355.

Two general strategies can be defined for the synthesis of glycopeptides. Glycosylated amino acids (as discussed above) can be incorporated into solid phase synthesis protocols with the respective advantages and drawbacks associated with this method. *See* Barany and Merrifield in Gross and Meinhoffer (eds) *The Peptides,* Vol. 2, Academic Press, New York, 1980, p. 1. Alternatively, peptides can be directly glycosylated. *See* Shier (1971) Proc. Natl. Acad. Sci. USA 68:2078.

Construction of the N-glycosyl linkage of glycopeptides usually proceeds through synthesis of a glycosyl amine followed by coupling with the side chain of aspartic acid. *See* Mark et *al.* (1963) Biochem. J. 87:274; and Bolton and Jeanloz (1963) J. Org. Chem. 28:3228). Numerous methods for synthesis of this amide bond have been utilized; these include carbodiimide (*see* Sheehan and Hess (1955) J. Am. Chem. Soc. 77:1067); 2-ethoxy-N-(ethoxycarbonyl)-1,2-dihydroquinoline (EEDQ) (*see* Dunstan and Hough (1974) Carbohydr. Res. 23:17 and Belleau and Malek (1968) J. Am. Chem. Soc. 90:1651); mixed and symmetrical anhydrides (*see* Coutsogeorgopoulos and Zervas (1961) J. Am. Chem. Soc. 83:1885; Garg and Jeanloz (1979) Carbohydr. Res. 70:47); acid chlorides (*see* Bergmann and Zervas (1932) Chem. Ber. 65:1201; and Ponpipom *et al.* (1980 Carbohydr. Res. 82:141); Woodward's reagent (*see* Woodward *et al.* (1961) J. Am. Chem. Soc. 832:1010; and Lanzilotti *et al.* (1964) J. Am. Chem. Soc. 86:1880); BOP (*see* Castro *et al.* (1975) Tetrahedron Lett. 1210); and HBTU (*see* Knorr *et al.*(1989) Tetrahedron Lett. 30:1927). An alternative which avoids the intermediacy of glycosyl amines involves condensation of the per-O-acetyl glycosyl isothiocyanate with carboxylic acids. *See* Khorlin *et al.* (1980) Carbohydr. Res. 85:201.

DCC-HOBt activation has been used to prepare a variety of glycopeptides in the solid phase. *See* Paulsen *et al.* (1988) Angew. Chem. Intl. Ed. Engl. 27:1365; Bardaji *et al.* (1988) Angew. Chem. Intl. Ed. Engl. 27:1365; Jannson et *al.* (1990) Tetrahedron Lett. 6991; Kunz and Unverzagt (1988) Angew. Chem. Intl. Ed. Engl. 27:1365; and Peters *et al.* (1991) Tetrahedron Lett. 5067. However, difficulties associated with a solid phase approach have greatly hindered development in this area. More specifically, orthogonal protecting groups typically must be used to incorporate the glycoamino acids into peptide synthesis formats. These protecting groups must be removable under essentially neutral conditions, because of the sensitivity of glycopeptides and glycoamino acids to extremes of pH. In addition, selective protecting group removal can be a key problem in elaborating either or both the carbohydrate or amino acid portion of the glycoamino acid.

Moreover, the stability of glycosidic bonds to the conditions of solid phase peptide synthesis is an important consideration. The acetal nature of glycosidic bonds make acid sensitivity a continual concern (*see* Kunz (1984) Nachr. Chem. Tech. Lab. 32:11). Two problems exist: cleavage of the glycosidic bond and isomerization of the anomeric center. Although the N-glycoside linkage is more acid and base stable than the O-glycoside bond, the lability of glycosidic bonds depends strongly on glycopeptide structure; some glycopeptides have survived treatment with HF; while in other examples the glycosidic bond has been cleaved with TFA. The O-glycosidic bond is, unlike the N-glycoside linkage, sensitive to basic conditions with pH ranges above 10 not tolerated (*see* Marshall and Nueberger, A (1970) Adv. Carbohydr. Chem. Biochem. 25:407). The rate of elimination depends, as expected, on the structure of the glycoconjugate; particularly important are the substituents on the amino and carbonyl groups with esterification or amidification greatly increasing the rate of elimination.

However, a solid phase synthesis format is particularly desirable since it is amenable to the practical generation of large libraries of novel, unnatural, and biologically-active polymers to be screened for compounds to serve as leads in drug discovery projects or as drugs in their own right. *see* U.S.. Patent No. 5,143,854; Serial No. 805,727, filed December 6, 1991; 07/624,120, filed December 6, 1990; 07/946,239, filed September 16, 1992; 762,522, filed September 18, 1991; 07/978,940, filed November 19, 1992; 07/971,181, filed November 2, 1992; each of which is

incorporated herein by reference for all purposes. These methods provide rapid and efficient means to synthesize polymers that are bio-compatible, i.e., compounds that are non-toxic and readily absorbed, and ideally that are synthesized from monomers available in large quantity, with a reasonable shelf life, optical activity, high-fidelity coupling chemistry, and stable to various chemical reagents used for protecting and deprotecting various side chains. Thus, it would be advantageous to have a method for synthesizing N-linked glycoconjugates, and particularly glycoamino acids and glycopeptides, in the solid phase. The present invention satisfies this and other needs.

SUMMARY OF THE INVENTION

The present invention provides methods for the solid-state synthesis of N-linked glycoconjugates which allow for the introduction of one or more saccharide moieties at any given position of a glycoconjugate or library of glycoconjugates, comprising the steps of:

a) on a surface of the substrate, providing an activated glycosyl acceptor; and

b) contacting the activated glycosyl acceptor with a glycosylating agent having a reactive functionality; a coupling catalyst; and optionally an exogenous base,

wherein a substrate-bound glycoconjugate is formed. This embodiment is particularly preferred for the synthesis of glycoconjugates, or libraries thereof, having a pendant or side chain carbohydrate moiety or moieties.

In a preferred aspect of this embodiment, the step of providing an activated glycosyl acceptor on the surface of the substrate further comprises the steps of:

i) on the surface of a substrate, providing a latent glycosyl acceptor bearing a protecting group;

ii) removing the protecting group to provide the latent glycosyl acceptor; and

iii) activating the latent glycosyl acceptor to provide an activated glycosyl acceptor. Preferably, the latent glycosyl acceptor comprises a carboxyl group and the step of activating the latent glycosyl acceptor comprises the step of converting the carboxyl group to an -OPfp ester.

In an alternative embodiment, the method comprises the steps of:

a) on a surface of the substrate, providing a glycosylating agent having a reactive functionality;

b) contacting the glycosylating agent with an activated glycosyl acceptor; a coupling catalyst; and optionally an exogenous base, wherein a substrate-bound glycoconjugate is formed. Another aspect of this invention provides for arrays of glycoconjugates produced using the above method.

According to another aspect of this latter embodiment, which is particularly preferred for the synthesis of linear glycoconjugates, the activated glycosyl acceptor comprises a monomer having a first functional group bearing a first protecting group and an activated carboxyl group and the method further comprises the steps of:

c) removing the first protecting group to yield the first functional group; and

d) repeating the steps of:

1) coupling a second monomer to the first functional group, the second monomer having a second functional group bearing a second protecting group; and

2) removing the second protecting group,

until the substrate-bound glycoconjugate is prepared.

Preferably, the activated glycosyl acceptor will comprise an activated carboxyl group; more preferably, an -ODhbt, -OPfp or -OSu ester; and most preferably, an -OPfp ester. In a particularly preferred embodiment, the carboxyl group comprises a side chain carboxyl of Asp or Glu. Preferably, the glycosylating agent comprises a glycosylamine and the reactive functionality will comprise a primary or secondary amino group. Accordingly, this method provides a means for producing N-linked glycoconjugates, including N-linked glycoamino acids and N-linked glycopeptides.

Preferably, the glycosyl acceptor will comprise the limiting reagent and the ratio of glycosyl acceptor to glycosylating agent is from about 1:2 to 1:5, and more preferably from about 1:3 to 1:4.

In a preferred embodiment, the exogenous base will comprise a tri(lower alkyl)amine, and more preferably, DIEA. Preferably, the ratio of glycosyl acceptor to exogenous base is from about 1:1 to 1:5,

more preferably from about 1:1 to 1:3, and most preferably about 1:2. In a preferred embodiment, the coupling catalyst will comprise HOBt. Preferably the ratio of glycosyl acceptor to coupling catalyst is from about 1:1 to 1:10, more preferably from about 1:3 to 1:8, and most preferably, about 1:5.

A further aspect of this invention provides for arrays of immobilized N-linked glycoconjugates and methods for preparing these arrays. The arrays are synthesized by

a) providing streptavidin in a predefined region of a substrate;

b) contacting a glycosylating agent having a reactive functionality; a glycosyl acceptor, wherein the glycosyl acceptor comprises an activated ester of a biotin-derivative; a coupling catalyst; and an exogenous base, to form a biotinylated glycoconjugate; and

c) contacting the biotinylated glycoconjugate with the substrate-bound streptavidin.

In a preferred embodiment, the activated ester of a biotin-derivative comprises biotinamidocapronamide. More preferably, the activated ester comprises an OSu-ester. Typically, the solid support will comprise a support having a plurality of wells, such as a 96-well microtiter plate.

Arrays of immobilized N-linked glycoconjugates can also be prepared by contacting an active ester-glycosylamide conjugate with a substrate-bound reactive functionality, optionally in the presence of a coupling catalyst such as HOBt. Typically, the active ester-glycosylamide conjugate is prepared by treating a glycosylamine with an active ester having two activated carboxyl groups, such as ethyleneglycol *bis*(succinimidyl succinate) or disuccinimidyl suberate.

Additional aspects of this invention provide for the cleavage of the various compounds which can be prepared by the methods described herein from the solid support on which they had been prepared.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides synthetic protocols for the amination of carbohydrates (Panel A) and the immobilization of aminated carbohydrates (Panel B).

Figure 2 illustrates a method for determining extent of glycoconjugate formation utilizing periodate cleavage followed by hydrazone formation and subsequent treatment with a fluorescent dye.

Histograms from a FACS analysis are also shown. The positive control (bottom histogram) is lactosylamide-beads. The middle histogram shows the presence of both free acid and glycoconjugate whereas only glycoconjugate is present in the top histogram.

Figure 3 illustrates a method for determining extent of glycoconjugate formation in a tagged library utilizing FACS analysis and WGA-fluorescein, with and without chitobiose present as a competing ligand. Histograms from the FACS analysis performed at each step are also shown.

Figure 4 provides representative lectin stains for beads treated with digoxigenin-labeled MAA (sialic acid specific lectin). Bound lectin was detected by means of an anti-digoxigenin/peroxidase conjugate and subsequent development. 1) TentaGel; 2) maltosyl-TentaGel; 3) sialyllactosyl-TentaGel; 4) lactosyl-TentaGel.

Figure 5 provides flow cytometry histograms of sugar-bead conjugates incubated with fluorescently labeled WGA. The center histogram shows the results from incubating a pool of glycosylamide resins comprising maltose, siallyllactose, chitobiose, LacNAc, GlcNAc6SO_3^-, and GlcNAc6PO_3^{2-}. The upper right histogram is the result of incubating chitobiosylamide beads with fluorescein-WGA (a positive control). The upper left histogram is the result of incubating lactosylamide beads with fluorescein-WGA (a negative control).

Figure 6 provides ELISA data for a streptavidin coated microtiter plate and 41 biotinylglycosides (duplicate runs for each) wherein the biotinylglycoside library was screened against WGA. The sugar component of each biotinylglycoside is listed below the table.

Figure 7 shows the results of an IC_{50} determination employing a lectin binding assay with chitobiosylbiotin. Lectin incubation was in the presence of varying concentrations of free sugar. The graph plots absorbance at 405 nm versus concentration of carbohydrate in mol/L. Data are not normalized. The assay was done in duplicates and the error bars are shown.

Figure 8 provides a schematic representation of the use of exoglycosidase digestion to obtain structural information regarding immobilized glycoconjugates.

Figure 9 provides a schematic representation of the use of glycopeptides as vectors for delivering pharmaceutical agents through the blood-brain barrier.

Figure 10 illustrates a method for determining extent of biotinylation in a library utilizing FACS analysis and fluorescently labeled avidin-fluorescein Histograms from the FACS analysis performed at each step are also shown. The reaction was found to be quantitative by the amino-fluorescein assay (upper left, lower histogram shows the controls). Staining of a mixture of deallylated and biotinylated libraries with avidin-fluorescein conjugate gave excellent resolution of the two populations. Running the experiment without the negative control showed that the library is represented in one small peak (right, small histogram).

Figure 11 provides a schematic representation of the synthesis of H-Q[Gal]YGGFL-OH with the corresponding HPLC traces for each of the products.

Figure 12 illustrates, from top to bottom, the structures of lactose, lactosylamine, ethyleneglycol mono(lactosylamidosuccinate) mono(succinimidyl succinate) and mono(lactosylamido) mono(succinimidyl) suberate.

Figure 13 provides ELISA-data for the interaction of streptavidin neoglycoproteins with lectins. Chosen oligosaccharides are reported to bind to the employed lectins. Shorthand notations of the glycosylbiotin/lectin-pairs are given. The background binding (streptavidin without glycosylbiotin) is represented by the right, unlabeled pair of columns respectively.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The description of the invention is provided as indicated by the following outline. In addition, Section I provides for a glossary of terms to facilitate the description of the invention. A number of terms and abbreviations are defined to have the general meanings indicated as used herein to describe the invention.

OUTLINE

I. Terminology

Unless otherwise stated, the following terms used in the specification and claims have the meanings given below:

"Activation" or "activating agent" refers to a reagent or energy source which selectively converts a latent glycosyl acceptor (preferably a carboxyl group) to an activated glycosyl acceptor (preferably an activated ester) which is capable of coupling to a glycosylating agent to generate a glycoconjugate linkage. For example, the activation of a

carboxyl group to produce the corresponding -OPfp ester comprises treatment with DCC and pentafluorophenol (*see, e.g.*, Kisfaludy and Schon (1983) Synthesis 325-327) or the trifluoroacetate derivative of pentafluorophenoxide and pyridine (*see* Green and Berman (1990) Tetrahedron Lett. 31:5851-5852).

"Activated ester" refers to an ester capable of reacting with an amine group to produce an amide linkage. Typically, the carbonyl carbon of an activated ester possesses a higher degree of positive charge character than the carbonyl carbon of an unactivated ester, i.e., a lower alkyl ester.

"Activated glycosyl acceptor" comprises a functional group which is capable of coupling with a glycosylating agent to generate a glycoconjugate linkage or with another nucleophile (e.g., a hydroxyl group, thiol group, or primary or secondary; amine) to produce an ester, thioester, or amide bond.

"Amino" or "amine group" refers to the group -NR'R", where R' and R" are independently selected from the group consisting of hydrogen, alkyl, substituted alkyl, aryl, substituted aryl, aryl alkyl, substituted aryl alkyl, heteroaryl, and substituted heteroaryl. In a primary amino group, both R' and R" are hydrogen, whereas in a secondary amino group, either, but not both, R' or R" is hydrogen.

An " α–amino acid" consists of a carbon atom, called the α-carbon, to which is bonded an amino group and a carboxyl group. Typically, this α–carbon atom is also bonded to a hydrogen atom and a distinctive group referred to as a "side chain." The hydrogen atom may also be replaced with a group such as alkyl, substituted alkyl, aryl, substituted aryl, heteroaryl, and other groups. The side chains of naturally occurring amino acids are well known in the art and include, for example, hydrogen (as in glycine), alkyl (as in alanine (methyl), valine (isopropyl), leucine (sec-butyl), isoleucine (iso-butyl), and proline (-$(CH_2)_3$-)), substituted alkyl (as in serine (hydroxymethyl), cysteine (thiomethyl), aspartic acid (carboxymethyl), asparagine, arginine, glutamine, glutamic acid, and lysine), aryl alkyl (as in phenylalanine, histidine, and tryptophan), substituted aryl alkyl (as in tyrosine and thyroxine), and heteroaryl (as in histidine). *See, e.g.*, Harper *et al.* (1977) Review of Physiological Chemistry, 16th Ed., Lange Medical Publications, pp. 21-24.

In addition to naturally occurring side chains, the amino acids used in the present invention may possess synthetic side chains. A "synthetic side chain" is any side chain not found in a naturally occurring

amino acid. For example, a synthetic side chain can be an isostere of the side chain of a naturally occurring amino acid. Naturally occurring and synthetic side chains may contain reactive functionalities, such as hydroxyl, mercapto, and carboxy groups. One skilled in the art will appreciate that these groups may have to be protected to carry out the desired reaction scheme. As stated above, the hydrogen at the α-carbon can also be replaced with other groups; those of skill in the art recognize the medicinal importance of α-methyl amino acids and other α–, α'–disubstituted amino acids.

"Aryl" or "Ar" refers to an aromatic carbocyclic group having a single ring (e.g., phenyl) or multiple condensed rings in which at least one ring is aromatic, (e.g., 1,2,3,4-tetrahydronaphthyl, naphthyl, anthryl, or phenanthryl), which can optionally be unsubstituted or substituted with, e.g., halogen, lower alkyl, lower alkoxy, lower alkylthio, trifluoromethyl, lower acyloxy, and hydroxy. However, according to the instant invention, the aromatic ring bearing the amide side chain cannot be further substituted with halogen. In addition, the aromatic ring bearing the amide side chain cannot possess a lower alkyl group ortho to the hydroxyl group (i.e., meta to the amide side chain). Preferred aryl groups include phenyl, 1-naphthyl, and 2-naphthyl.

"Carboxy" refers to the group -R(COOH) where R is alkyl, substituted alkyl, aryl, substituted aryl, aryl alkyl, substituted aryl alkyl, heterocyclic, heteroaryl, or substituted heteroaryl.

"Ester" refers to the group -COOR where R is alkyl, substituted alkyl, aryl, substituted aryl, aryl alkyl, substituted aryl alkyl, heterocyclic, heteroaryl, or substituted heteroaryl.

"Exogenous base" refers to a nonnucleophilic organic base, for example, a tri(lower alkyl)amine, such as diisopropylethylamine or triethylamine.

"Functional group" or "functionality" refers to a portion of a molecule which, under selected circumstances, performs a desired coupling or cleavage reaction with another moiety. Such coupling may be via covalent, or other types of bonds. Examples of functional groups will be readily appreciated by those in the art and include, but are not limited to, amino groups, hydroxyl groups, thiol groups, carboxyl groups, carbonyl groups, and the like.

"Glycoamino acids" refer to glycosylated amino acids, e.g., amino acids bearing a carbohydrate moiety.

"Glycoconjugates" refer to glycoamino acids, glycopeptides, glycoproteins, or other conjugates comprising a saccharide covalently bonded to a functional group via a glycoconjugate linkage.

"Glycoconjugate linkage" refers to an amide bond for N-linked glycoconjugate and an ether bond for O-linked glycoconjugates.

"Glycopeptides" or "glycoproteins" are biopolymers which contain one or more carbohydrate chains linked covalently to a peptide backbone.

"Glycuronic acid" refers to an oxidation product of a carbohydrate in which the primary alcohol group is oxidized to carboxyl.

"Heterocycle" refers to a saturated, unsaturated, or aromatic carbocyclic group having a single ring (e.g., morpholino, pyridyl or furyl) or multiple condensed rings (e.g., naphthyridinyl, quinoxalyl, quinolinyl, indolizinyl or benzo[b]thienyl) and having at least one hetero atom, such as N, O or S, within the ring, which can optionally be unsubstituted or substituted with, e.g., halogen, lower alkyl, lower alkoxy, lower alkylthio, trifluoromethyl, lower acyloxy, and hydroxy. The term "heteroaryl" or "HetAr" refers to a heterocycle in which at least one heterocyclic ring is aromatic.

"Hydroxyl" or "hydroxy" refers to the group -OH.

"Latent glycosyl acceptor" comprises a functional group which upon activation is capable of coupling with a glycosylating agent.

"Limiting reagent" refers to that substance which limits the maximum amount of product formed in a chemical reaction, no matter how much of the other reactants remains.

"Lower alkyl" refers to an alkyl group of one to six carbon atoms. Lower alkyl groups include those exemplified by methyl, ethyl, n-propyl, i-propyl, n-butyl, t-butyl, i-butyl (2-methylpropyl), cyclopropylmethyl, i-amyl, n-amyl, and hexyl. Preferred lower alkyls are methyl, ethyl, n-propyl, i-propyl, and n-butyl. If more than one alkyl group is present in a given molecule, then each may be independently selected from "lower alkyl" unless otherwise stated.

"Monomer" refers to a member of the set of small molecules which can be joined together to form a polymer, especially those having an inherent polarity. The set of monomers includes but is not restricted to, for example, the set of common L-amino acids, the set of D-amino acids, the set of synthetic or natural amino acids, th set of nucleotides and the set of pentoses and hexoses. As used herein, monomers refers to any

member of a basis set for synthesis of a polymer. For example, dimers of the 20 naturally occurring L-amino acids form a basis set of 400 monomers for synthesis of polypeptides. Different basis sets of monomers may be used at successive steps in the synthesis of a polymer. Those of skill in the art will recognize that a "monomer" is simply one type of "chemical building block" and that any type of chemical building block can be employed in the present method, regardless of whether one is synthesizing an oligomer or a small organic molecule or some other molecule.

"Oligomer" or "polymer" refers to a compound formed from the chemical or enzymatic addition of monomer subunits or building blocks. Such oligomers include, for example, both linear, cyclic, and branched polymers of nucleic acids, polysaccharides, phospholipids, and peptides having either α-, β-, or γ-amino acids, heteropolymers in which a known drug is covalently bound to any of the above, polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, polyacetates, or other polymers which will be readily apparent to one skilled in the art upon review of this disclosure. Polysaccharides, for example, refer herein to a carbohydrate which can be hydrolyzed into many monosaccharides. Polynucleotides refer to molecules containing a series of nucleotide monomers. One of skill in the art will appreciate that if the monomer subunits are chemical building blocks, the resulting polymer will comprise a smal organic molecule.

"Peptide" refers to a polymer in which the monomers are alpha amino acids and which are joined together through amide bonds, alternatively referred to as a polypeptide. In the context of this specification it should be appreciated that the amino acids may, for example, the L-optical isomer or the D-optical isomer. Peptides are often two or more amino acid monomers long, often 4 or more amino acids long, often 5 or more amino acids long, often 10 or more amino acids long, often 15 or more amino acids long, and often 20 or more amino acid monomers long, for example. Standard abbreviations for amino acids are used (e.g., P for proline). These abbreviations are included in Stryer, Biochemistry, Third Ed., 1988, which is incorporated herein by reference for all purposes.

"Polar solvent" refers to a protic or aprotic liquid that is either ionic or has a large permanent dipole moment. Examples of polar solvents include DMSO and water.

"Predefined region" refers to a localized area on a substrate which is, was, or is intended to be used for formation of a selected glycoconjugate and is otherwise referred to herein in the alternative as a "selected" region. The predefined region may have any convenient shape, e.g., circular, rectangular, elliptical, wedge-shaped, etc. For the sake of brevity herein, "predefined regions" are sometimes referred to simply as "regions." In some embodiments, a predefined region and, therefore, the area upon which each distinct polymer sequence is synthesized is smaller than about 1 cm^2 or less than 1 mm^2. Within these regions, the glycoconjugate synthesized therein is preferably synthesized in a substantially pure form. In additional embodiments, a predefined region can be achieved by physically separating the regions (i.e., beads, resins, gels, etc.).

"Protecting group" refers to a chemical group that exhibits the following characteristics: (1) reacts selectively with the desired functionality in good yield to give a derivative that is stable to the projected reactions for which protection is desired; 2) can be selectively removed from the derivatized substrate to yield the desired functionality; and 3) is removable in good yield by reagents compatible with the other functional group(s) generated in such projected reactions. Examples of protecting groups can be found in Greene *et al.* (1991) Protective Groups in Organic Synthesis, 2nd Ed. (John Wiley & Sons, Inc., New York). Preferred protecting groups include photolabile protecting groups (such as methylpiperonyloxycarbonyl (Menpoc), methylpiperonyl (Menp), nitroveratryl (Nv), nitroveratryl-oxycarbonyl (Nvoc), or nitroveratryloxymethyl ether (Nvom)); acid-labile protecting group (such as Boc or DMT); base-labile protecting groups (such as Fmoc, Fm, phosphonioethoxycarbonyl (Peoc, *see* Kunz (1976) Chem. Ber. 109:2670); groups which may be removed under neutral conditions (e.g., metal ion-assisted hydrolysis), such as DBMB (*see* Chattopadhyaya *et al.* (1979) I.C.S. Chem. Comm. 987-990), allyl or alloc (*see, e.g.,* Greene and Wuts, "Protective Groups in Organic Synthesis", 2nd Ed., John Wiley & Sons, Inc., New York, NY (1991), 2-haloethyl (*see* Kunz and Buchholz (1981) Angew. Chem. Int. Ed. Engl. 20:894), and groups which may be removed using fluoride ion, such as 2-(trimethylsilyl)ethoxymethyl (SEM), 2-

(trimethylsilyl)ethyloxycarbonyl (Teoc) or 2-(trimethylsilyl)ethyl (Te) (*see, e.g.*, Lipshutz *et al.* (1980) Tetrahedron Lett. 21:3343-3346)); and groups which may be removed under mild reducing conditions (e.g., with sodium borohydride or hydrazine), such as Lev. *Id. at* 30-31, 97, and 112.

Particularly preferred protecting groups include Fmoc, Fm, Nvoc, Nv, Boc, CBZ, allyl, alloc, Npeoc (4-nitrophenethyloxycarbonyl) and Npeom (4-nitrophenethyloxy-methyloxy).

"Reactive functionality" or "reactive site" refers to a portion of a molecule which, under selected circumstances, performs a desired coupling with another moiety, preferably to form a glycoconjugate linkage. Preferably, the reactive site will be a primary amino group or carboxyl group or other nucleophile, such as a hydroxyl group or thiol group, or secondary amino group.

"Saccharide" or "sugar" refers to any of a group of water-soluble carbohydrates of relatively low molecular weight. Monosaccharides are those which cannot be hydrolyzed into a simpler form and are based on the general formula $C_x(H_2O)_y$. Disaccharides are carbohydrates which yield two molecules of the same or of diferent monosaccharides when hydrolyzed. Oligosaccharides are those which yield 2-6 monosaccharide units on hydrolysis. Polysaccharides yield more than six monosaccharides on hydrolysis. For purposes of the present invention, the terms "saccharide" and "sugar" are intended to include monosaccharides, disaccharides, oligosaccharides, and polysaccharides. Standard abbreviations for saccharides are used (e.g., Glu for glucose). These abbreviations are included in"Abbreviated Terminology of Oligosaccharide Chains, IUPAC Recommendations 1980" as found in J. Biol. Chem. 257:3347-3351 (1982), which is incorporated herein by reference for all purposes. Trivial name employed herein include: lactose (Lac) for Gal(β1-4)Glc; maltose for Glc(α1-4)Glc; chitobiose for GlcNAc(β1-4)GlcNAc; sialyllactose for Neu5Ac(α2-3)Gal(β1-4)Glc; and LacNAc for Gal(β1-4)GlcNAc.

"Substrate" or "support" refers to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many embodiments, at least one surface of the substrate will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different polymers with, for xample, wells, raised regions, pins, etched trenches, or the like. According to other embodiments, the substrate(s) will take the form of beads, resins, gels,

microspheres, or other geometric configurations. The substrate is alternatively referred to herein as a support.

"Weak acid" refers to a substance having a pK_a between about 0.1 and 8, and preferably between about 0.1 and 5. Preferred weak acids include trifluoroacetic acid, acetic acid, citric acid, ascorbic acid, lactic acid, and the like.

Isolation and purification of the compounds and intermediates described herein can be effected, if desired, by any suitable separation or purification procedure such as, for example, filtration, extraction, crystallization, column chromatography, thin-layer chromatography, thick-layer (preparative) chromatography, distillation, or a combination of these procedures. Specific illustrations of suitable separation and isolation procedures can be had by references to the examples hereinbelow. However, other equivalent separation or isolation procedures can, of course, also be used.

Abbreviations: The following abbreviations are intended to have the following meanings:

Boc = t-butyloxycarbonyl
BOP = benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate
DCC = dicyclohexylcarbodiimide
Ddz = dimethoxydimethylbenzyloxy
DMT = dimethoxytrityl
Fmoc = fluorenylmethyloxycarbonyl
HBTU = 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt = 1-hydroxybenzotriazole hydrate
HODhbt = 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine
HOPfp = pentafluorophenol
HOSu = N-hydroxysuccinimide
Menpoc = methylpiperonyloxycarbonyl
Menp = methylpiperonyl
NV = nitroveratryl
Nvoc = 6-nitroveratryloxycarbonyl and other photoremovable groups
PG = protective group
TFA = trifluoroacetic acid

II. Description of the Invention

A. Overview

The present invention provides an approach to the solid-state synthesis of N-linked glycoconjugates which allows for the introduction of one or more saccharide moieties at any given position of a glycoconjugate or library of glycoconjugates. An activated glycosyl acceptor is contacted with a glycosylating agent having a reactive functionality; a coupling catalyst; and optionally an exogenous base to form a substrate-bound glycoconjugate. To produce branched N-linked glycoconjugates, typically, the glycosyl acceptor is immobilized prior to the coupling reaction. This strategy is also applicable to the production of glycoconjugates wherein the carbohydrate moiety comprises the terminus or resides in the backbone of the glycoconjugate. The synthesis of linear N-linked glycoconjugates can be readily accomplished by immobilizing the glycosylating agent on the solid substrate.

According to a preferred embodiment, the glycosyl acceptor will comprise the -OPfp ester of a carboxyl group. The reactive site preferably will comprise a primary or secondary amino group. Thus, this method provides a means for producing N-linked glycoamino acids and N-linked glycopeptides. The described method is compatible with conventional peptide synthesis, protection, and deprotection protocols.

B. The Solid Substrate

The solid substrate is of the type commonly used in peptide and polymer synthesis. Such substrates are composed of materials such as glass, latex, heavily cross-linked polystyrene or similar polymers, gold or other colloidal metal particles, and other materials known to those skilled in the art. Except as otherwise noted, the chemically reactive groups with which such solid supports may be derivatized are those commonly used for solid phase synthesis of the polymer and thus will be well known to those skilled in the art, i.e., carboxyls, amines and hydroxyls. A preferred substrate is glass, as described in U.S. Patent No. 5,143,854, *supra*. Another preferred substrate is resin, such as the beads described in co-pending U.S. Patent Application Serial No. 07/946,239, filed September 16, 1992, *supra*. Other preferred substrate is BIAcore chips which have an oxidized, free

carboxyl-containing dextran layer and magnetic beads, e.g., Dynal beads. A particularly preferred solid substrate is SASRIN resin, available from Bachem.

The substrate may also have linker or spacer molecules that separate the synthesized polymers from the substrate surface, usually to facilitate receptor interaction during screening. The linker molecules preferably have lengths sufficient to allow the polymers to which they are bound to interact freely with any molecules exposed to the substrate surface, such as synthetic reagents or receptors which are an object of study, and they will include typically a functional group to which a monomer is attached at the start of the polymer synthesis. Typically prior to the start of polymer synthesis, the functional group bears a protecting group, such as a photoremovable protecting group.

The choice of functionality used for binding the glycosyl acceptor or glycosylating agent to the substrate will depend on the nature of the compound to be synthesized and the type of substrate. Conditions for coupling monomers and polymers to solid substrates through a wide variety of functional groups are known in the art. *See, e.g.,* U.S. Patent No. 4,542,102; U.S. Patent No. 4,282,287;Merrifield, "Solid Phase Peptide Synthesis," J. Am. Chem. Soc., (1963) 85:2149-2154; Geysen *et al.*, "Strategies for Epitope Analysis Using Peptide Synthesis," J. Imm. Meth., (1987) 102:259-274; Pirrung *et al.*, U.S. Patent No. 5,143,854; and Fodor *et al.*, "Light-Directed Spatially-Addressable Parallel Chemical Synthesis," Science (1991) 251:767-773, each of which is incorporated herein by reference.

C. The Glycosyl Acceptor

1. Immobilized Glycosyl Acceptor

According to the present invention, an activated glycosyl acceptor is coupled to a glycosylating agent. According to one embodiment, the glycosyl acceptor is attached to the substrate. Typically the glycosyl acceptor will be introduced to and immobilized upon the substrate by coupling a monomer or polymer comprising the glycosyl acceptor (typically in its latent form or a protected derivative thereof) to the substrate. Thus, the monomer or polymer that is attached to the substrate surface will include a functionality which can covalently bind the molecule to the substrate (e.g., an activated carbonyl, acyl halide, or activated hydroxyl) as well as the glycosyl acceptor, typically in a latent

form or a protected derivative thereof (i.e., the latent glycosyl acceptor, optionally bearing a protecting group). Typically, this strategy will be employed for the synthesis of glycoconjugates wherein the carbohydrate moiety is attached to a side chain of the molecular backbone or to the terminus of the molecule.

According to another aspect of this invention, the solid support will be derivatized such that a carboxyl group is at the surface (e.g., BIAcore chips) and thus, the latent glycosyl acceptor will comprise this surface carboxyl group.

2. Soluble Glycosyl Acceptor

According to another embodiment, the glycosylating agent is attached to the substrate and the glycosyl acceptor is introduced in a soluble form. Typically, this protocol will be utilized to produce linear glycoconjugates (i.e., wherein the carbohydrate moiety resides within the molecular backbone).

3. Soluble Glycosyl Acceptor and Glycosylating Agent

In a further embodiment, the coupling of the glycosylating agent and the glycosyl acceptor will occur in the solution phase. The resulting glycoconjugate is then immobilized on the solid surface for further derivatization or screening. Preferably, the highly specific association of biotin to streptavidin is exploited as a means to immobilize the glycoconjugate. This embodiment will find use in the production of glycoconjugate libraries for screening and is discussed in greater detail below.

4. Protecting Groups

If the glycosylating agent possesses additional reactive sites, it may be necessary to protect them during the coupling reaction or other stages of the glycoconjugate synthesis. Suitable protecting groups include acid-labile, base-labile, photoremovable, or removable under neutral conditions. *See, e.g.,* Green, Protecting Groups in Organic Synthesis, Wiley 1985, pp. 218-288, which is incorporated herein by reference. The choice of a particular protecting group will be determined generally by the conditions under which the polymer is formed and by the types of protecting groups used on the side chains of the other monomers to be used in polymer synthesis. In a most preferred embodiment, the

protecting groups are photoremovable and their removal is accomplished by exposing the surface or selected regions thereof to light (e.g., from a light source through a mask) or removable under neutral conditions. Such protecting groups and techniques are described in U.S. Patent No. 5,148,854 and co-pending U.S. Patent Applications Serial No. 07/624,120, filed December 6, 1990, and 07/971,181, filed November 2, 1992.

5. Preferred Glycosyl Acceptor

Typically, the latent glycosyl acceptor will comprise a carboxyl group. In a preferred embodiment, the activated glycosyl acceptor will comprise an activated side chain carboxyl group or terminal carboxyl group of an amino acid or peptide. In a particularly preferred embodiment, the latent glycosyl acceptor will comprise a carboxyl group of a dicarboxylic amino acid residue. The other functionalities of the residue are optionally protected with a suitable protecting group(s). For example, one carboxyl group of the monomer is coupled to either the substrate or to a monomer or polymer which has been previously bound to the substrate. The other carboxyl group (the latent glycosyl acceptor) is protected with a photolabile protecting group or a protecting group which can be removed under neutral conditions, preferably the allyl group. The amino group typically will be protected with a suitable protecting group, for example Fmoc or Boc. Most preferably, the latent glycosyl acceptor will comprise the side chain carboxyl group of Asp or Glu.

The latent glycosyl acceptor is activated prior to the coupling reaction. Preferably, the activated glycosyl acceptor will comprise a -ODhbt, -OSu or -OPfp ester. More preferably, the activated glycosyl acceptor will comprise an -OSu or -OPfp ester. Most preferably, the activated glycosyl acceptor will comprise an -OPfp ester.

D. The Glycosylating Agent

1. Soluble and Immobilized Glycosylating Agents

According to one aspect of the invention, a soluble glycosylating agent is coupled to a substrate-bound activated glycosyl acceptor. Alternatively, a substrate-bound glycosylating agent is coupled to a soluble glycosyl acceptor. According to this aspect, the glycosylating agent often is introduced to the substrate in a latent form. Specifically, an unprotected glycuronic acid or glyconolactone is coupled to a substrate-bound amino group using conventional HBTU/HOBt activation to

produce the substrate-bound glycosylating agent. Preferably, the substrate-bound amino group will comprise an amino group of a substrate-bound amino acid or peptide.

2. Protecting Groups

Although the glycosylating agent possesses additional reactive sites, since the coupling reaction typically occurs at the latter stage of a synthetic sequence, these sites need not be protected. If further derivatization is to occur and protection of the reactive sites is necessary, the protecting groups can be introduced prior or subsequent to the coupling. As with the glycosyl acceptor, suitable protecting groups include acid-labile, base-labile, photoremovable, or removable under neutral conditions. *See, e.g.,* Green, Protecting Groups in Organic Synthesis, Wiley 1985, pp. 218-288, which is incorporated herein by reference. The choice of a particular protecting group will be determined generally by the conditions under which the polymer is formed and by the types of protecting groups used on the side chains of the other monomers to be used in polymer synthesis.

3. Preferred Glycosylating Agent

Generally, the methods described herein will utilize a glycosylamine as the glycosylating agent and thus, the reactive functionality will comprise a primary or secondary amino group. A variety of glycosylamines are commercially available.

In addition, almost any carbohydrate can be converted to the corresponding glycosylamine using the synthetic protocols shown in Figure 1 and described in detail below, as well as methods known in the art. *See* Likhosherstov *et al.* (1986) Carbohydr. Res. 146:C1-C5. In a particularly preferred embodiment, an amino group is introduced at the reducing end of the substrate-bound sugar using excess ammonium carbonate in a polar solvent, preferably, DMSO or water, to form the substrate-bound glycosylating agent.

The amination reaction typically yields stereochemically well defined products. An N-acetyl group or an equatorial hydroxyl function in the 2-position of the substrate directs the anomeric amino function of the product into the β-configuration. The stereoselectively is typically >95% enantiomeric excess. The extent of conversions of the saccharides to the glycosylamines are typically 80 to 95%.

Another embodiment of this invention provides for a method for producing diglycosylamines by treating the glycosylamine with a coupling catalyst, such as HOBt, in a polar solvent, preferably DMSO. Typically, between about 0.3 and 0.8, preferably between about 0.4 and 0.6, and most preferably about 0.5 equivalents of the coupling agent is used.

E. The Reaction Conditions

The coupling reaction is generally performed at ambient conditions. However, depending on the nature of the glycosylating agent and the glycosyl acceptor, one of skill in the art will recognize that it may be necessary to perform the coupling at temperatures other than ambient. Typically, the coupling reaction can be accomplished between about 0°C and 100°C.

Preferably, the glycosyl acceptor will comprise the limiting reagent and the ratio of glycosyl acceptor to glycosylating agent is from about 1:2 to 1:5, and more preferably from about 1:3 to 1:4.

The coupling reaction is performed in the presence of a coupling catalyst.

In a preferred embodiment, the coupling catalyst will comprise HOBt, HOAt (1-hydroxy-7azabenzotriazole *see* Carpino (1993) J. Am. Chem. Soc. 115:4397-4398), or other coupling catalysts used for making amide bonds. Typically, the ratio of glycosyl acceptor to coupling catalyst is from about 1:1 to 1:10, more preferably from about 1:3 to 1:8, and most preferably, about 1:5.

According to some embodiments, an exogenous base is employed in the coupling reaction. The base serves to suppress side product formation, to stabilize the pH and thus, protect any acid-sensitive functionality, and to ensure that any salts which may be present are neutralized Typically, exogenous base will be utilized in the synthesis of glycoamino acids and glycopeptides. In a preferred embodiment, the exogenous base will comprise a tri(lower alkyl)amine and more preferably, DIEA. Typically, at least one equivalent of base is used and generally, the base will comprise $\leq$ 10% of the reaction solution. Preferably, the ratio of glycosyl acceptor to exogenous base is from about 1:1 to 1:5, more preferably from about 1:1 to 1:3, and most preferably about 1:2.

When the activated glycosyl acceptor comprises a -OPfp ester, the extent of coupling can be followed semi-quantitatively by monitoring

the release of the pentafluorophenoxide ion which is a highly chromogenic species.

The methods of the present invention are readily automated using technology presently available for binding and reacting monomers to form polymer chains and removing the byproducts of those reactions. Moreover, the methods described herein are amenable to the simultaneous production of a variety of different glycoconjugates having one or more different saccharide moieties at any given position.

The methods described herein proceed with retention of the stereochemistry at the anomeric center and can be characterized as having a high percent asymmetric synthesis or percent enantiomeric excess where these terms serve as a measure of the extent to which one enantiomer is produced in excess over the other. (For example, a reaction sequence that produces 59% of one isomer and 41% of another isomer would have a percent asymmetric synthesis or percent enantiomeric excess of 59 - 41% = 18%). Typically, the reaction sequence described herein will exhibit at least about a 60% percent enantiomeric excess overall; preferably, at least about a 70% percent enantiomeric excess; more preferably, at least about a 80% percent enantiomeric excess; and even more preferably, at least about an 90% percent enantiomeric excess, where a perfectly stereospecific reaction sequence would have a 100% percent enantiomeric excess. *See, e.g.*, Morrison and Mosher, "Asymmetric Organic Reactions", 2nd Ed., American Chemical Society, Washington, D.C. (1976), which is incorporated herein by reference. Moreover, the reaction conditions are sufficiently mild that even complicated saccharides and amino acids can be safely converted to the corresponding glycoconjugate.

Removal of the substrate-bound glycoconjugates, if desired, can be accomplished by techniques known in the art. For example, the compound can be attached to the substrate surface by a cleavable linker such as those described in U.S. Serial No. 07/978,940, filed November 19, 1992 and U.S. Patent No. 5,242,974, incorporated herein by reference. Typically, because of the sensitive nature of the glycosidic bond, mild acidic conditions, typically using a weak acid or a dilute solution of a strong acid, and preferably 1-50% TFA, and more preferably 1-10% TFA, is used to cleave the glycoconjugates from the substrate. More concentrated solutions, typically 50% TFA, will be used to remove acid-cleavable protecting groups from the glycoconjugate.

F. Libraries

1. Encoded Synthetic Libraries

a. Synthesis

The methods described above may be used to prepare and screen large numbers of compounds, in the hundreds, the thousands and even the ten thousands in a reasonable period of time. Synthesis may be combined with screening in various different ways to screen compounds in unusually large libraries. Preferably, the techniques described above are used to synthesize more than 2, preferably more than 5, preferably more than 10, more preferably more than 50, more preferably more than 100, and more preferably more than 1,000 different molecules simultaneously. It is understood, however, that the term "single compound" or "single molecule" as used herein includes different regio and stereo isomers of that compound. Also, the term "single compound" or "single molecule" does not mean that only one copy of that compound or molecule is attached to each support. Rather, multiple copies of *that* compound or molecule can be included on the support.

In a preferred embodiment, the latent glycosyl acceptors (i.e., typically, carboxyl groups) are present on resin beads, and the substrate-bound compounds are constructed using a stochastic method of polymer synthesis as described in co-pending U.S. Patent Application Serial No. 07/946,239, filed September 16, 1992 which is a continuation-in-part of U.S. Patent Application Serial No. 07/762,522, filed September 18, 1991.

In general, such methods comprise apportioning the supports comprising either the glycosyl acceptor or the glycosylating agent among a plurality of reaction vessels; exposing the supports in each reaction vessel under conditions wherein the glycoconjugate is formed and wherein said glycoconjugate is different for each of the reaction vessels; and optionally, pooling the supports.

b. Example of a Specific Embodiment

Using this technique, a peptide library of approximately 100,000 members, each having the structure Ac-X X E(OAl) X P - resin, where X represents any of the 18 side chain protected amino acids Ala, Arg(Pmc), Asn(Trt), Asp(OtBu), Gln, Glu(OtBu), Gly, His(Trt), Leu, Lys(Boc), Met, Phe, Pro, Ser(tBu), Thr(tBu), Trp(Boc), Tyr(tBu), and Val (the abbreviations in parentheses refer to the various protecting groups used

on the side chain of each amino acid) was assembled in an automated fashion. The solid-phase glycosylation strategy centered upon the selective removal of the allyl ester (OAl) protecting group and subsequent condensation of the acid function with an unprotected glycosylamine.

A first amino acid monomer selected from the group listed above, each protected with an Fmoc group on the amino group, was separately coupled to 90 mg beads. After washing to remove excess reagents and byproducts, the beads were pooled and then divided between several reaction vessels, each containing a different amino acid monomer (selected from the list given above). The amino groups of the bound amino acid monomers were deprotected and separately coupled to the free carboxyl group of the soluble amino acid monomers to form bound dimers. The beads were washed and again reapportioned between vessels a second time. This sequence of reaction and random reapportionment can be continued until sequences having the desired lengths are formed. For the given example, two rounds of monomer coupling were conducted. To each of the dipeptides was then coupled an allyl protected Glu. Two additional rounds of amino acid coupling were then conducted.

The glycosyl acceptor was then produced by deprotecting and activating the sidechain carboxyl group on the Glu residue which is common to each of the molecules. Typically, $Pd(PPh_3)_4$ was used to remove the allyl group and to liberate the free-carboxyl group. Activation to form the glycosyl acceptors can be accomplished using means known in the art, and preferably, via treatment with pentafluorophenyl trifluoracetate. A first set of glycosylating agents, as described above, was then introduced to the various aliquots of resin-beads-containing-surface reactive functionality to yield a library of glycoconjugates. Aliquots of the original 18^4-member library were diversified by conjugation to 17 different glycosylamines (Gal, Fuc, GlcNAc, Glc(α1-4)Glc, Gal(β1-4)GlcNAc, Glc6NAc, $Gal6SO_3^-$, $GlcN2,6(SO_3^-)_2$, GalA, GalNAc, $GlcNAc3SO_3^-$, Glc(β1-4)Glc, Glc3NAc, Man(α1-3)Man, AllNAc, ManNAc, and Lyx).

To assess the extent of deallylation and subsequent condensation, a fluorescence assay was developed to indicate presence or absence of the free carboxylate on the otherwise fully protected, immobilized peptide. After conversion of the δ-carboxylate of the library into a pentafluorophenyl active ester (OPfp ester), a fluorescein derivative containing a free primary amine group on a short spacer arm was reacted with the library. The library was then subjected to FACS analysis which

rapidly assessed the extent of fluoresceination of each library component individually and thus, gave an estimate for the completeness of library conversion. For example, a sample of the OPfp-activated library was split into two fraction. One half was glycosylated and the other aliquot was mixed with a negative control (Ac-X X E (OAl) X X -resin). Both fractions were incubated with the fluorescein amine and analyzed using a FACS instrument. The glycopeptide library fluoresced as a single, narrow peak at the same low intensity as the negative control. This results establishes the quantitative and general nature of the glycosylamine condensation.

c. Assays

The -OPfp esters are surprisingly stable and thus, the beads can be reapportioned either prior or subsequent to deprotection and activation. For example, a mixture of deprotected, but unactivated beads and deprotected and activated beads can be separated by fluorescence activated bead sorting using a probe comprising a fluroescein derivative carrying a primary amine on a spacer. (Flow cytometry, commonly referred to as fluorescence activated cell sorting or FACS should be viewed as equivalent to "fluorescence activated molecular sorting" or "fluorescence activated bead sorting" for purposes of the present invention.) The dye 4'-((aminoacetamido)methyl)fluorescein (available from Molecular Probes, Eugene, Oregon) gave superior resolution as compared to fluoresceinamine and 5-((5-aminopentyl)thioureidyl)fluorescein. Peak identification was performed by running the deprotected and the activated libraries separately as shown. For a typical experiment, 10,000 events were accumulated with a laser setting of 488 nm and a filter of 530 ± 30 nm.

The amino-fluorescein probe described above can also be used to assess the degree of library derivatization. More specifically, the activated library is titrated with various amounts of ethylamine and subsequently probed for remaining OPfp-esters using the amine-dye. In the range of 30 to 3 ethylamine molecules/OPfp-ester, the library shifts from dark to bright. At ratios above or below this range, the library is either dark or bright. The library typically migrates as a single sharp peak, indicating that the derivatization is not sequence specific.

FACS analysis can also be utilized to ascertain the degree of glycosylation. More specifically, after the coupling reaction, th

glycoconjugate is subjected to periodate oxidation which converts vicinal diols into aldehydes. Treatment with 5-(((2-(carbohydrazino)methyl)thio)acetyl)aminofluorescein yields the corresponding hydrazone. A FACS instrument to sort the beads and to identify and isolate physically individual beads showing high fluorescence as shown in Figure 2. See Methods in Cell Biology, Vol. 33 (Darzynkiewicz, Z. and Crissman, H.A., eds., Academic Press); and Dangl and Herzenberg, 1982, J. Immunol. Meth. 52:1-14, both incorporated herein by reference. This experiment was conducted with the library described above. The character "X" in the figure represents a random amino acid selected from those listed above. "MB" symbolizes the microbead.

d. Tagging

In a preferred aspect of this embodiment, each solid support is tagged with an identifier tag that can be easily decoded to report the compounds formed on the solid support. The tag can be directly attached either to the solid support or the tag can be included on the compound itself. In this latter embodiment, cleavage of the compound from the solid support will still permit identification of the compound. Each of these embodiments is disclosed in International Patent Application Publication No. WO 93/06121.

According to this embodiment, the resin beads were first differentiated with a cocktail of Fmoc-Thr(tBu)-OH/HATU and DMT-oxybutyrate-OSu. The ratio of Fmoc/DMT on the beads was photometrically determined to be 243:1. The 3'-end of the oligonucleotide was first constructed. The first pair of amino acids were added, as were two sets of three nucleotides, each set of which encodes for a particular amino acid. Then Fmoc-E(OAl)-OH was coupled without any encoding, as this residue was common to all beads. Synthesis was completed by addition of the last two scrambled positions with the corresponding codons for the tags and construction of the 5'-tail of the oligonucleotide. The allyl group was then removed and the side chain carboxyl group was activated. Acetamidoglucosylamine was coupled to the glycosyl acceptor to yield the library of tagged glycoconjugates

The tagged library was probed using a lectin binding assay. See Figure 3. The glycoconjugates were mixed and incubated with wheat germ agglutinin (WGA)-fluorescein (also referred to herein as fluorescently-labeled WGA). WGA is a lectin with specificity for

acetamidoglucose (GlcNAc) and exhibits a dissociation constant for GlcNAc of 7.6×10^{-4}, for chitobiose of 4.9×10^{-5}, and chitotriose of 1.2×10^{-5}. Recently, WGA has been shown to also bind oligo-N-acetyllactosaminoglycans. In this group, recognition is associated with the GlcNAcβ(1-6)Gal epitope rather than the GlcNAcβ(1-3) isomer. *See* Renkonen *et al.* (1988) Biochem. Cell Biol. 66:449-453. Under this assay, the library stained brightly. More importantly, the library exhibited specific binding which could be blocked by the addition of soluble ligand. Thus, binding of the lectin was not impaired by the presence of oligonucleotide.

Still another method for incorporating a tag with the solid support is disclosed in U.S. Patent Application Serial No. 08/146,886, filed November 2, 1994, which application is incorporated herein by reference in its entirety.

2. Glycobeads

The present methods allow for the immobilization of almost any given oligosaccharide through the formation of a glycoconjugate. Specifically, if the solid surface bears a glycosyl acceptor (preferably an activated ester), the glycosylamine corresponding to almost any carbohydrate can be immobilized using the present methods. For example, the aminated sugar can be directly coupled to the oxidized, free carboxyl-containing dextran layer of a BIAcore chip.

According to a preferred embodiment, the glycosylamine is formed and coupled to an amino-functionalized support by means of a bifunctional spacer (e.g., disuccinimidylsuberate (DSS)). Preferably, a large excess (typically at least 5 equivalents based on amine and preferably at least 10 equivalents based on amine) DSS is used. The resulting glycosylamide-active ester can then be immobilized on for example, amino-functionalized resins, such as ethyleneglycol-grafted polystyrene resin (TentaGel). Alternatively, a thiol functionality can be introduced into the carbohydrate with succinimidyl-S-acetylthioacetate (SATA). The thio-saccharide derivative can then be coupled to a bromoacetylated support (employing hydroxylsuccinimidylbromoacetate (HSBA))

Success of the immobilization reaction can be monitored using immunostaining (e.g., with the Glycan Detection Kit, available from Boehringer Mannhein, *see* Haselbeck and Hoesel in *Protein Glycosylation: Cellular Biotechnological and Analytical Aspect*, GBF Monographs,

Volume 15, Conrate (ed.), Verlag Chemie, Weinheim (1990)). Periodate oxidation of the bonded sugars yields aldehydes that react with a digoxigenin labeled (digoxigenin-succinyl-aminocaproic acid hydrazide). An alkaline phosphatase/anti-digoxigenin conjugate binds irreversibly to the oxidized carbohydrates and is followed by a precipitative color reaction (indoylphosphate and nitro blue tetrazolium).

The resulting sugar-resin conjugates also can be probed using a lectin binding assay. Typically, a population of glycoconjugates immobilized on beads are treated with maackia amurensis agglutinin (MAA). The assay involves incubation with digoxigenin-labeled lectin and detection with anti-digoxigenin-alkaline phosphatase and a precipitative stain. *See, e.g.*, Haselbeck *et al.* (1990) Anal. Biochem. 191:25-30. This lectin is specific for Neu5Ac(α2-3)Gal and unambiguously recognizes the epitope in the ligand sialyllactose. An example of results from this assay format is shown in Figure 4.

According to another embodiment, the glycosylamide active esters, typically the glycosylamide-N-hydroxysuccinimidyl ester derivatives, are separately conjugated to 10 μm diameter, monodisperse polystyrene resin. *See, e.g.*, Needels *et al.* (1993) Proc. Natl. Acad. Sci. USA 90:10700-10704. The sugar-bead suspensions are then subjected to fluorescence activated particle analysis as described above using a fluorescently-labeled lectin. Those sugar-bead conjugates showing selective lectin binding can then be determined. An example of this assay procedure using the saccharides maltose, siallyllactose, chitobiose, LacNAc, $GlcNAc6SO_3^-$ and $GlcNAc6PO_3^{2-}$ is shown in Figure 5. Only a fraction of the assayed beads exhibited lectin binding to fluorescently-labeled WGA. This subpopulation consisted of chitobiosylamide beads exclusively, as inferred by assaying each sugar-bead conjugate individually.

3. Glycoplates

A particularly preferred embodiment of this invention provides for the production of an array of substrate-bound glycoconjugates suitable for use as a research tool, for example as a substrate for the performance of assays, for investigating carbohydrate/protein interactions, for determining lectin or selectin specificities, and for developing carbohydrate based drugs. The glycoconjugate library typically is produced on a solid substrate having a plurality of sections, preferably in a 96-well microtiter plate. Microtiter plates having wells covered with covalently

immobilized streptavidin are commercially available (Pierce). A spectrum of saccharides were aminated and subsequently coupled to biotin bearing a glycosyl acceptor, typically N-hydroxysuccinimidyl biotinamidocaproate (Pierce). Typically, an excess of glycosyl acceptor is used. Preferably, at least 2.5 equivalents based on glycosylamine, and more preferably about 5 equivalents, of N-glycosyl acceptor is used. These biotinylated glycoconjugates were introduced into the streptavidin-coated microtiter plates and incubated for several hours. The biotin-streptavidin binding is highly specific and thus, the carbohydrates are displayed on the surface of a protein with low non-specific binding.

Plate functionality was proven by lectin binding assays as shown in Figure 6. Sugars, immobilized in wells, were detected with digoxigenin-labeled lectins and anti-digoxigenin/alkaline phosphatase conjugate as described above. The library contained three known ligands for the lectin: GlcNAc (bar #7 in Figure 6); chitobiose (bar #12 in Figure 6); and GlcNAcβ(1- 6)Gal (bar #19 in Figure 6). Negative controls (not shown) were as low as the background.

This assay format was also employed to determine IC_{50} values for free WGA-ligands. The assay was performed with chitobiosylbiotin and lectin incubation was in the presence of varying concentrations of free sugars. Figure 7 illustrates the rank order of ligand affinities, which were as expected.

4. VLSIPS™ Libraries

In another embodiment, the VLSIPS™ technique is used to construct a library of glycoconjugates for screening with various active substances (see U.S. Patent No. 5,143,854). Such a library of different compounds can be simultaneously screened for receptor binding or some other activity.

A predefined region of a glass substrate having latent glycosyl acceptors bearing photolabile protecting groups is exposed to light shone through a mask to form regions of latent glycosyl acceptors and protected glycosyl acceptors. All or only a selected portion of the surface-bound glycosyl acceptors can be deprotected. The deprotected, substrate-bound glycosyl acceptors are activated and contacted with a first set of glycosylating agents as described above. After washing to remove excess reagents and byproducts, the same or different regions of the substrate are exposed to light to form free reactive functionality on the surface.

Another predefined region of the substrate is then deprotected by exposure to light and the above-described sequence of introduction, activation, and bond formation are repeated until the desired library of surface-bound compounds is prepared. Thus, using this technique, libraries of N-linked glycoconjugates can be produced

5. Other Library Formats

Libraries also can be prepared using the pin approach developed by Geysen *et al.*, for combinatorial solid-phase peptide synthesis. A description of this method is offered by Geysen *et al.*, (1987) J. Immunol. Meth. 102:259-274, incorporated herein by reference. According to this method as it may be practiced in the present invention, a series of 96 pins are mounted on a block in an arrangement and spacing which correspond to a 96-well Microtiter reaction plate, and the surface of each pin is derivatized to contain terminal aminomethyl groups. The pin block is then lowered over a series of reaction plates in sequence to immerse the pins in the wells of the plates where coupling occurs at the terminal aminomethyl groups and the various reactions in the reaction schemes described above are performed.

Reagents varying in their substituent groups occupy the wells of each plate in a predetermined array, to achieve as ultimate products a unique glycoconjugate on each pin. By using different combinations of substituents, one achieves a large number of different compounds with a common central glycoconjugate subunit . Once formed in this manner, each derivative may be cleaved from its pin by treatment with acid, as described herein.

6. Secondary Modifications

a. Glycosyltransferases

The glycoportion of an immobilized glycoconjugate can be secondarily modified via the bond-forming action of glycosyltransferases or hydrolases or other chemical means. The reaction(s) will be performed typically on resin with the glycosylating agent or modifying agent (e.g., the glycosyltransferase) present in excess. In a preferred embodiment, a hydrolase whose action is to be reversed is used. The resulting products are glycoconjugates with glycoportions that are not readily available from biological sources, non-naturally occurring, and/or not easily accessible via solution chemistry.

b. Exoglycosidases

Exoglycosidases have been well characterized in terms of activity and specificity and are commercially available from a variety of suppliers. Exoglycosidases can be obtained which are specific for the type of sugar forming the non-reducing end of an oligosaccharide and for the alpha/beta stereoisomerism of their glycosidic bond. Depending on the enzyme's origin, specificity can also include the penultimate sugar and its binding hydroxyl group. Thus, a further embodiment of this invention which exploits the fact that the methods described herein produce immobilized glycoconjugates with the non-reducing ends of the sugar free and involves the introduction of further diversity into the glycoconjugate library by treatment of the library with an exoglycosidase(s).

In addition, since each exoglycosidase will only digest its corresponding sugar, treatment immobilized conjugates with a panel of exoglycosidases will provide structural information on the glycoconjugates. Binding of a receptor to the exoglycosidase treated library yields information regarding the type of sugar forming the reducing end of the epitope; the number of sugars masking the epitope; and the type of sugar marking the link between the masking and binding region.

For example, lacto-N-fucopentaose III, Gal(β1-4)Fuc(α1-3)GlcNAc(β 1–3)Gal(β 1-4)Glc (available from Oxford Glycosystems) is immobilized according to the methods described above and is then subjected to repeated enzymatic digestion with a set of exoglycosidases. Three rounds of digestion with nine exoglycosidases (*see* Figure 8) are conducted with each performed such that a portion of the immobilized glycoconjugate is spared from treatment. Immunostaining with digoxigenin labeled WGA-lectin will stain sections 9-16, indicating that fucose masked the epitope (for binding occurred after fucosidase action); that the epitope's non-reducing end is N-acetylglucosamine; and that this GlcNAc is bound via a beta-glycosidic linkage to the rest of the saccharide (for binding disappeared after treatment with β-N-acetylglucosaminidase).

In addition, exoglycosidase digestion can be employed in combination with surface plasmon resonance detection. *See* Malmqvist (1993) Nature 361:186. The resulting sensorgram allows for the direct read-out of the oligosaccharide sequence.

G. Uses of Glycoconjugates and Libraries Thereof

1. Library Screening

The glycoconjugate libraries (or secondarily diversified libraries) will find a variety of uses. Among the uses of these compounds will be their screening for binding with a receptor. More specifically, libraries of glycopeptides will find use in the identification of epitopes recognized by receptors, such as the Selectins involved in cell-cell adhesion phenomena. In addition, the isolation of antibodies directed against the carbohydrate portion of an invading organism's (such as a virus or bacteria) surface glycopeptides and the observation of their binding to glycopeptides synthesized according to the methods described herein will allow for the identification of complementary carbohydrate structures and thus, the development of new vaccines against the invading organism. Alternatively, microorganisms often make use of the host cell's glycosylation pattern to cling to specific cell types. *See* Lingwood (1992) Curr. Opin. Struct. Biol. 2:693. Screening against the virus's receptor will yield oligosaccharide leads for the neutralization of hostile microorganisms. An example of such a phenomena has been shown in the inhibition of HIV adsorption to target cells by derivatized polysaccharides. *See* Bârzu *et al.* (1993) J. Med. Chem. 36:3546.

Libraries of these glycoconjugates can be screened to identified those glycoconjugates having the desired activities. Screening of peptides, for example, to determine their affinity with a receptor is discussed in detail in PCT application No. WO 90/15070, previously incorporated by reference herein.

2. Drug Delivery

The glycoportion of glycopeptides has also been shown to have an effect on biodistribution. *See* Rasmussen (1992) Curr. Opin. Struct. Biol. 2:682. For example, the binding of carbohydrates to specific cell surface receptors can be exploited for site-directed drug delivery. The specific targeting of mannose-grafted liposomes to macrophages (*see* Meddan *et al.* (1993) Biotechnol. Appl. Biochem. 17:37) and the uptake of galactosylated DNA complexes by hepatocytes via the asialoglycoprotein receptor (*see* Haensler and Szoka (1993) Bioconjugate Chem. 4:85) are examples of this approach.

In addition, glycosylation of a given peptide (using the methods described herein) can increase the hydrophilicity of the peptide.

One consequence of this modification is improved solubility. But more importantly, since glycosylation inhibits clearance via the liver biliary pathway and switches the drug to the slower renal secretion pathway, glycosylation of the peptide will also result in enhanced serum half-life of the peptide. *See also,* Fisher *et al.* (1991) J. Med. Chem. 34:3143. Screening of libraries of glycopeptides will allow for the identification of glycoconjugates having the desired drug delivery properties.

The glyco portion of a glycopeptide can also serve as a "tag" for site-specific drug delivery. Specifically, lectins have been shown to bind to the glycocalix of the endothelium of cerebral blood vessels and transcytose through the cerebral endothelium. Thus, proteins capable of binding to the glycocalix and simultaneously to glycosylated pharmacophores displaying the same carbohydrate epitope can be used as a vector or shuttle for delivering the glycosylated pharmacophores through the blood-brain barrier. *See* Figure 9. Screening of libraries of glycopeptides will allow for the identification of these vectors.

H. Other Applications

One of skill in the art will appreciate that the coupling procedure described herein can be utilized to produce compounds other than glycoconjugates.

More specifically, the glycosyl acceptor can be reacted with nucleophiles other than amino-sugars (i.e., glycosylating agents). For example, coupling of the glycosyl acceptor with virtually any compound having a free primary or secondary amino group, such as an amino acid, amino-nucleotide, or other amine containing compound, hydroxyl group, or thiol group can be accomplished.

More specifically, a library of glycosyl acceptors (the OPfp actived peptide library described above) was activated and subsequently reacted with biotinamidopentylamine. The presence of the biotin moiety was probed with a fluorescently labeled protein, avidin. Here a FACS-shift of two orders of magnitude was observed for the biotinylated versus the allyl protected library. The two populations were well separated, indicating complete and homogeneous biotinylation of the library. The reaction was found to be quantitative by the amino-fluorescein assay as shown in Figure 10.

A library of activated glycosyl acceptors has also been coupled to lisinopril, a potent competitor inhibitor of angiotensis converting

enzyme, via its primary amino group. When, as in the case of an amino acid, the nucleophile contains other functionality, such as a carboxyl group, that functionality may be protected prior to coupling of the glycosyl acceptor to the nucleophile.

In addition, the glycosyl acceptor can be reacted intramolecularly with a free reactive functionality, typically a primary or secondary amino group, hydroxyl group, or thiol group, to form a cyclized polymer (see co-pending U.S. Patent Application Serial No 07/978,940, filed November 19, 1992 and U.S. Patent No. 5,242,974. In one preferred embodiment, the glycosyl acceptor and the free reactive functionality, typically, an amino group, reside in the same polymer chain and thus, upon activation and coupling, a loop in the polymer between those two positions is created. The loop may comprise the entire polymer (for example, where the glycosyl acceptor is attached to the first subunit of the polymer and the free reactive functionality is attached to the last subunit) or some portion thereof. According to some aspects of this embodiment, the free reactive functionality will reside on the side chain of the amino acid. In other aspects, the free reactive functionality will comprise a secondary amino group, e.g., an amino group of the polymer backbone. In an alternative embodiment, the substrate-bound compound is formed via the coupling of a free reactive site at one position of the substrate with a glycosyl acceptor at a second, proximate position on the substrate.

Coupling of the glycosylating agent can be performed as described above. The coupling reaction should, however, be performed under conditions effective to cause formation of an internal cyclic bond, as known in the art. The primary factors contributing to the efficiency of the cyclization reaction include accessibility of the terminus of the polymer to the cyclization site, the efficiency of the cyclization process, the selectivity of the cyclization towards the terminal monomer unit or other desired monomer unit, the type of activation required to achieve cyclization and the stability of the new bond formed.

In addition to forming cyclized polymers, the methods of the present invention are adaptable to reversed-polarity polymer synthesis, wherein a previously exposed terminus of the polymer is attached directly or indirectly to the substrate, while a previously directly attached terminus or portion of the polymer is placed in an exposed position (see co-pending U.S. Patent Application Serial No. 07/978,940, filed November 19, 1992 and U.S. Patent No. 5,242,974). In one such adaptation, a tether with a first site

is used to anchor a tether molecule, directly or indirectly using linker molecules, to a substrate, preferably by a covalent linkage. A second site on the tether is used to synthesize the polymer with a first polarity. A third site, typically the glycosyl acceptor, is used to cyclize the polymer by bonding a portion of the polymer, having a reactive functionality, to the third site. Finally, the reversal of polarity is achieved by breaking the bond between the second site and the polymer.

EXAMPLES

The following examples are included for the purpose of illustrating the invention and are not intended to limit the scope of the invention in any manner.

I. Synthesis of Glycosylamines

A. Aqueous Method

A solution of the saccharide (1%, weight by volume (w/v)) in saturated aqueous ammonium carbonate was stirred at room temperature for 5 days. Solid $(NH_4)_2CO_3$ (ca. 40 mg/mg saccharide) was added in fractions during the course of the reaction to ensure saturation. Kinetics were followed by thin layer chromatography (TLC , elution with 1-propanol:ethyl acetate:water, 6:1:3, detection with orcinol- and ninhydrin reagents, respectively). After the conversion, samples were frozen and lyophilized for three days. Gravimetrically determined yields were typically in the order of 100→110%. Samples showing yields >150% were discarded, others were repeatedly lyophilized.

The crude glycosylamines were used without purification for acylation reactions in solution. For solid-phase glycosylations, excess solid $(NH_4)_2CO_3$ was most effectively removed by dissolving the crude glycosylamine in warm methanol (ca. 70°C). After termination of CO_2-evolution, the methanol was slowly evaporated and the residual material dried *in vacuo*.

^1H NMRs of glycosylamines were run in D_2O and conversions were found in the range of 50→90%. However, by comparing D_2O-spectra with d_6-DMSO-spectra for identical samples, it became apparent that the hydrolytic stability of different glycosylamines differed greatly. In D_2O, the half-life of some glycosylamines fell within the time frame of a ^1H NMR experiment (5 min.), making the lower conversions artifactual. A striking example is the difference between the 1-amines of lactose and 2'-methyl lactose. Both reactions exhibited 90% conversions when monitored in d_6-DMSO. In D_2O, the lactosylamine gave the same result, but its 2'-methyl derivative showed 50% hydrolysis.

B. DMSO Method

Glycosylamines of superior quality for use in solid-phase glycosylatior were obtained when the amination was performed in DMSO. Solutions of the

saccharide (5% w/v) in saturated ammonium carbonate/DMSO were stirred for 8→48 hours. Solid $(NH_4)_2CO_3$ was added in intervals to keep the solutions saturated. After filtration, the saccharides were precipitated by addition of acetone or acetone/ether. The recovered solids were washed with acetone and dried *in vacuo*. Glycosylamines prepared by this method were analyzed by proton-NMR in deutero-DMSO and conversions were found to be quantitative (>90%). Specific analytical data for representative glycosylamines prepared according to the above method are summarized below.

SACCHARIDE	m/z[1]	m/z[2]	^{1}H-NMR[3]
Lyx[4]	N.D.	N.D.	4.019 ppm (d, 1.2 Hz)
Ara	No reaction		
Glc[5]	N.D.	N.D.	4.093 ppm (8.7 Hz)
Gal[6]	N.D.	N.D.	4.035 ppm (8.7 Hz)
Fuc[7]	N.D.	N.D.	4.023 ppm (8.7 Hz)
Man	N.D.	N.D.	4.343 ppm (s)
Rib	No reaction		
Xyl	149/150.1 ($M+H^+$)	150.0766/150.0761	4.169 ppm (d, 9.1 Hz)
GlcNAc[8]	N.D.	N.D.	4.156 ppm (d, 9.0 Hz)
GalNAc	220/221.1($M+H^+$)	221.1138/221.1142	4.081 ppm (d, 9.4 Hz)
ManNAc	220/221.2 ($M+H^+$)	221.1137/221.1135	N.D.
Glc-6-NAc	220/221.0 ($M+H^+$)	221.1138/221.1133	4.074 ppm (d, 8.8 Hz)
Glc-3-NAc	220/221.0 ($M+H^+$)	221.1138/221.1135	4.177 ppm (d, 8.7 Hz)
Neu5Ac	N.D.	N.D.	N.D.
MurNAc	N.D.	N.D.	N.D.
Gal-6-Me	No reaction		

1 Calculated/found by low resolution mass spectroscopy.
2 Calculated/found by high resolution mass spectroscopy.
3 For the N-linked anomeric proton (δ and J).
4 Obtained from Toronto Research Chemicals.
5 Obtained from Sigma.
6 Obtained from Sigma.
7 Obtained from Sigma.
8 Obtained from Sigma.

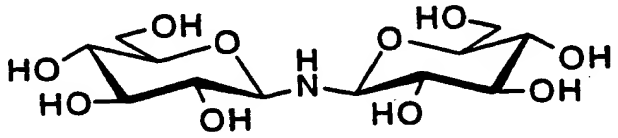
Glc-3-Me	No reaction		
All-2-NAc	220/221.0 ($M+H^+$)	221.1137/221.1139	4.385 ppm (d, 9.6 Hz)
All-3-NAc	220/221.0 ($M+H^+$),	N.D.	4.294 ppm (d, 9.4 Hz)
GlcA	215/238.0 ($M_{sodium\ salt}+Na^+$)	N.D.	4.111 ppm (d, 8.8 Hz)
GalA	N.D.	N.D.	4.032 ppm (d, 8.8 Hz)
GlcNAc-3-sulfate	N.D.	N.D.	4.281 ppm (d, 9.4 Hz)
GlcNAc-6-sulfate	322/345.1 ($M_{sodium\ salt}$)	N.D.	4.192 ppm (d, 8.8 Hz)
GlcNAc-6-phosphate	344/345.1 ($M_{disodium\ salt}$)	N.D.	4.169 ppm (d, 8.8 Hz)
GlcN-2,3-disulfate	382/405.1 ($M_{disodium\ salt}$)	N.D.	4.226 ppm (d, 8.8)
GlcN-2,6-disulfate	382/405.1 ($M_{disodium\ salt}$)	N.D.	4.221 ppm (d, 8.4 Hz)
Man-6-phosphate	N.D.	N.D.	4.361 (s)
Gal-6-phosphate	N.D.	N.D.	4.066 ppm (d, 8.8 Hz)
Gal-6-sulfate	281/282.1 ($M_{sodium\ salt}+H^+$)	282.0260/282.0255,	4.068 ppm (d, 8.6 Hz)
Rib-5-phosphate	No reaction		
Glc(α1-4)Glc	341/342.1 ($M+H^+$)	342.1400/342.1391	4.117 ppm (d, 8.7 Hz)
Glc(α1-4)Glc(α1-4)Glc	503/504.2 ($M+H^+$)	N.D.	4.121 ppm (d, 8.8 Hz)
Glc(β1-4)Glc	341/342.1 ($M+H^+$)	342.1400/342.1407	4.125 ppm (d, 8.7 Hz)
Gal(β1-4)Glc	341/342.1 ($M+H^+$)	N.D.	4.129 ppm (d, 8.9 Hz)
Neu5Ac(α2-3)-Gal(β1-4)Glc	632/633.3 ($M_{sodium\ salt}+H^+$)	633.2354/633.2350	4.064 ppm (d, 9.1 Hz)
Neu5Ac(α2-6)-Gal(β1-4)Glc	654/655.2 ($M_{sodium\ salt}+H^+$)	655.2174/655.2170	4.023 ppm (d, 9.0 Hz)
Neu5Ac(α2-3)-Gal(β1-4)GlcNAc	695/696.2 ($M_{sodium\ salt}+H^+$)	696.2439/696.2391	N.D.
Fuc(α1-2)Gal(β1-4)Glc	487/488 ($M+H^+$)	488.1979/488.1975	4.103 ppm (d, 8.8 Hz)
Gal(β1-4)Fuc(α1-3)Glc	487/488 ($M+H^+$)	488.1979/488.1975	4.134 ppm (d, 8.8 Hz)
Gal-2-Me(β1-4)Glc	355/356 ($M+H^+$)	356.1557/356.1563	4.136 ppm (d, 8.3 Hz)

GlcNAc(β1-4)GlcNAc	423/424.2 ($M+H^+$)	424.1931/424.1930	4.157 ppm (d, 8.7 Hz)
Gal(β1-4)GlcNAc	382/383.2 ($M+H^+$)	383.1666/383.1661	4.189 ppm (d, 8.7 Hz)
GlcNAc(β1-6)GlcNAc	423/424 ($M+H^+$)	424.1931/424.1930	4.148 ppm (d, 9.4 Hz)
Gal(β1-3)GlcNAc	382/383 ($M+H^+$)	383.1666/383.1669	4.042 ppm (d, 8.7 Hz)
Gal(β1-6)GlcNAc	382/383 ($M+H^+$)	383.1666/383.1665	4.183 ppm (d, 9.4 Hz)
GlcNAc(β1-6)Gal	382/383 ($M+H^+$)	383.1666/383.1665	4.024 ppm (d, 8.7 Hz)
GalA(α1-4)GalA	369/370 ($M_{diacid}+H^+$)	N.D.	4.189 ppm (d, 8.8 Hz)
Gal(α1-4)Gal	N.D.	N.D.	4.104 ppm (d, 8.6 Hz)
Gal(β1-6)Gal	341/342.1 ($M+H^+$)	342.1400/342.1404	4.065 ppm (d, 8.9 Hz)
Gal(β1-3)GalNAc	382/383 ($M+H^+$)	N.D.	4.137 ppm (d, 9.0 Hz)
Gal(β1-4)Man	341/342.1 ($M+H^+$)	342.1400/342.1404	4.374 (s)
Gal(β1-3)Ara	311/312.1 ($M+H^+$)	312.1295/312.1300	4.165 (s)
Neu5Ac(α2-6)-Gal(β1-4)Glc-NAc(β1-3)-Gal(β1-4)Glc	N.D.	N.D.	4.120 ppm (d, 8.7 Hz)
Neu5Ac(α2-3)Gal(β1-3)Neu5Ac(α2-6)GalNAc	N.D.	N.D.	N.D.
Fuc(α1-4)Gal(β1-4)Fuc(α1-3)Glc	633/634.2 ($M+H^+$)	634.2558/634.2551	4.092 ppm (d, 8.9 Hz)
Gal(β1-3)Fuc(α1-4)GlcNAc(β1-3)Gal(β1-4)Glc	852/853.3 ($M+H^+$)	853.3301/853.3283	4.123 ppm (d, 8.7 Hz)
Man(α1-3)Man	341/342 ($M+H^+$)	342.1400/342.1407	4.361 ppm (s)
Neu5Ac(α-2-3)Gal(β1-4)Glc	N.D.	633.2354/633.2350 ($M_{sodium\ salt}+H^+$)	4.064 ppm (d, 9.1 Hz)
Neu5Ac(α-2-6)Gal(β1-4)Glc	N.D.	655.2174/655.2170 ($M_{sodium\ salt}+H^+$)	4.023 ppm (d, 9.0 Hz)
Neu5Ac(α-2-3)Gal(β1-4)GlcNAc	N.D.	696.2439/696.2391 ($M_{sodium\ salt}+H^+$)	N.D.

Neu5Ac(α-2-6) Gal(β1-4)GlcNAc (β1-3)Gal(β1-4)Glc	N.D.	N.D.	4.120 ppm (d, 8.7 Hz)

II. Synthesis of Diglycosylamines

To a solution of 1-hydroxybenzotriazole (56 mmol) in DMSO (0.04 mL) was added glucosylamine (Sigma, 20 mg, 112 mmol). The mixture was warmed until homogenous, cooled to room temperature and vortexed for 2.5 hours. Product was precipitated with acetone (1 mL) and recovered by centrifugation. The residue was washed with methanol/acetone (1:2) and ether. Yield: quantitative. FAB-MS: 341 (calcd.) 342.2 (found, $M+H^+$). ^{1}H-NMR: 4.900 ppm (0.13 H, 4.9 Hz, H-1α, unknown sideproduct), 4.296 ppm (0.74 H, 8.8 Hz, H-1β, product), 4.119 ppm (0.13 H, 8.6 Hz, H-1β, educt).



Structure of diglucosylamine

III. Synthesis of N-linked Glycopeptides

As a representative example, the synthesis of H-Q[Gal]YGGFL-OH is shown schematically (with the corresponding HPLC traces for each of the products) and described in Figure 11.

A. Preparation of Fmoc-E[OAll]Y[*t*Bu]GGFL-Sasrin (1)

The peptide was assembled by FastMoc™ chemistry (coupling: Fmoc-amino acid[9] (1 millimole (mmol)) in NMP (2.1 gram (g)) and a solution of HBTU/HOBt in DMF (2.2 g of 0.45 molar (M)) and diisopropylethylamine (DIEA, 0.22 g, 1.7 eq.); 27 minutes (min) at room

9 Fmoc-Glu-[O-Allyl]-OH and Fmoc-Asp-[O-Allyl]-OH are commercially available from Propeptide.

temperature (RT); Fmoc deprotection: 20% piperidine in NMP for 3 min at RT) on an ABI 431A-peptide synthesizer, starting from Fmoc-Leu-Sasrin (416 mg, 0.25 mmol, 0.6 mmol/g, available from Bachem).

B. Preparation of Fmoc-E[OH]Y[*t*Bu]GGFL-Sasrin (2)

To a solution of chloroform (5 milliliters (ml)), acetic acid (0.66 ml) and N-methylmorpholine (0.32 ml) under nitrogen atmosphere was added $Pd(PPh_3)_4$ (0.4 g, 0.32 mmol), Fmoc-E[OAl]Y[*t*Bu]GGFL-Sasrin (0.2 g, 0.12 mmol) and PPh_3 (0.4 g, 1.53 mmol). The mixture was vortexed for 2 hours at room temperature and the resin recovered by filtration and washed with $CHCl_3$, N,N-dimethylformamide (DMF) and methanol.

C. Preparation of Fmoc-E[OPfp]Y[*t*Bu]GGFL-Sasrin (3)

To a suspension of Fmoc-E[OH]Y[*t*Bu]GGFL-Sasrin (20 mg, 12 µmol) in anhydrous DMF (0.1 ml) were added pyridine (20 µl) and pentafluorophenyl-trifluoroacetate (40 µl, 0.23 mmol, available from Aldrich Chemical Co.). The mixture was vortexed for 2 hours at room temperature. The resin was recovered by filtration and washed with DMF and CH_2Cl_2.

D. Preparation of Fmoc-Q[Gal]Y[*t*Bu]GGFL-Sasrin (4)

To a suspension of Fmoc-E[OPfp]Y[*t*Bu]GGFL-Sasrin (20 mg, 12 µmol) in anhydrous dimethylsulfoxide (DMSO, 0.66 ml) were added galactosylamine (8 mg, 45 µmol, available from Sigma), DIEA (4 µl) and HOBt (8 mg, 59 µmol, available from ABI). The mixture was vortexed for 15 min at room temperature. The resin was recovered by filtration and washed with DMSO and CH_2Cl_2.

E. Preparation of H-Q[Gal]Y[*t*Bu]GGFL-Sasrin

A suspension of Fmoc-Q[Gal]Y[*t*Bu]GGFL-Sasrin (20 mg, 12 µmol) in 20% piperidine/DMF (0.4 ml) was vortexed for 15 min at room temperature. After filtration, the procedure was repeated and the recovered resin was washed with DMF and CH_2Cl_2.

F. Preparation of H-Q[Gal]YGGFL-OH (5)

A suspension of H-Q[Gal]Y[*t*Bu]GGFL-Sasrin (20 mg, 12 µmol) in 50% TFA/CH_2Cl_2 (1 ml) was vortexed for 15 min at room temperature. After filtration, the procedure was repeated and the resin was washed

extensively with CH_2Cl_2. The combined filtrates were concentrated *in vacuo*, and the product was precipitated with ethyl ether and isolated by centrifugation. After dissolution in acetonitrile/water and lyophilization the crude material was obtained as a white fluffy solid (6.4 mg, 8 µmol, 66%). Purification was achieved on a preparative HPLC-system (Beckman System Gold with an analog interface module 406, two solvent delivery modules 110B, a programmable detector module 166 and a RP C18 column (Waters RCM 25x10)). Solvent A: 0.1 % TFA in water, solvent B: 0.1% TFA in acetonitrile. A linear gradient (0→5 min at 10% B, 5→35 min to 50% B, 35→40 min to 95% B, 40→50 min at 10% B) was used at 4 ml/min. The sample was collected and lyophilized to give the desired product (5.6 mg, 7 µmol, 58%).

IV. Preparation of Additional N-linked Glycopeptides

Following the procedures set forth above, the following N-linked glycopeptides were prepared.

Product	m/z (calculated/found) low and high resolution		^{1}H-NMR for N-inked anomeric proton: d (ppm) and J (Hz)		Fraction in HPLC crude (%)
H-N(GalAβ)YGGFL-OH	845/846.2 ($M+H^+$)	846.3521/846.3534	4.757 (d)	8.7	37
H-N(Gal-6-sulfateβ)YGGFL-OH	911/910.4 ($M-H^+$)	910.3119/910.3140	4.747 (d)	7.8	51

H-N(GlcN-2,6-disulfateβ)YGGFL-OH	990/909.5 (M-SO_3-H^+) and 989.5 (M-H^+)[10]	909.3310/909.3300 and 989.2765/989.2868	4.684 (d)	9.6	41
H-N(Galβ)YGGFL-OH	831/854.7 (M+Na^+)	854.3548/854.3548	4.800 (d, 0.35H), 4.689 (d, 0.65H)[11]	8.4	92
H-NGlc(α1-4)Glc(α1-4)5(Glcβ)YGGFL-OH	1803/1804.7 (M+H^+)	N.D.	4.752 (d)	9.0	51
H-YGGFLN(Lyxα/β)-OH	801/802.2 (M+H^+)	802.3623/802.3639	5.044 (d, 0.60H) and 5.007 (d, 0.40H)	8.4 and 3.9	36 and 25
H-YGGFLN(GlcNAcβ)-OH	872/895.7 (M+Na^+)	895.3814/895.3822	4.778 (d)	9.6	99
H-YGGFLN(GalNAcβ)-OH	872/873.3 (M+H^+)	873.3994/873.3999	4.741 (d)	9.9	99
H-YGQ(GlcNAc-6-sulfateβ)GFL-OH	988/1011.6 (M+Na^+)	1011.3358 1011.3364	4.814 (d)	9.3	99

10 The desulfation is Fab-MS induced, HPLC showed no sign of the desulfated species.

11 The signal duplication is due to the existence of Z/E amide rotamers.

H-YGQ(GlcNAcβ)GFL-OH	886/909.8 ($M+Na^+$)	909.3970/909.3983	4.788 (d)	10.2	99
H-YGQ(Glcα1-4)(Glcβ)GFL-OH	1007/1008.7 ($M+H^+$)	1008.44141008.4428	4.715 (d)	9.0	95
H-YGQ(Glcβ4)(Glcβ)GFL-OH	1007/1008.5 ($M+H^+$)	1008.44141008.4419	4.710 (d)	9.3	92
H-YGQ(Galβ)GFL-OH	845/846.3 ($M+H^+$)	846.3885/846.3876	4.628 (d)	8.4	99
H-YGGFLQGlc(α1–4)Glc(α1–4)$_5$Glcβ)-OH	1817/1840.8 ($M+Na^+$)	N.D.	4.711 (d)	8.4	89
H-YGGFLQGlcβ-OH	845/868.7 ($M+Na^+$)	868.3705/868.3708	4.682 (d)	9.0	99
H-YGGFLQ(ManNAcβ)-OH	886/909.7 ($M+Na^+$)	909.3970/909.3961	5.076 (d)	9.3	91
H-YGGFLQGal(β1-4)(Manα)-OH	1007/1008.6 ($M+H^+$)	1008.44141008.4409	5.1047 (s)	0	76
H-Q(Gal-6-sulfateβ)YGGFL-OH	925/924.4 ($M-H^+$)	924.3279/924.3279	4.699 (d)	8.4	47
H-QFucβYGGFL-OH	829/852.4 ($M+Na^+$)	852.3756/852.3751	4.660 (d)	8.4	99
H-Q(GlcNAc(β1-4)(GlcNAcβ)–YGGFL-OH	1089/1090.6 ($M+H^+$)	1090.49451090.4953	4.813 (d)	9.3	92
H-Q(GlcNAcβ)YGGFL-OH	886/909.4 ($M+Na^+$)	909.3970/909.3944	4.825	9.6	99

H-QGal(β1–4)(Glcβ)YGGFL-OH	1007/1008.4 ($M+H^+$)	1008.44 141008.4399	4.730 (d)	9.0	95
H–Q-(Gal(β1–3)Fuc(α1–4)-GlcNAc(β1-3)Gal(β1-4)Glcβ)-YGGFL-OH	1518/1373.6 (M-Fuc+H^+) and 1519.7 ($M+H^+$)	N.D.	N.D.	N.D.[12]	36 and 41

V. Analytical HPLC

For analytical purposes a fraction (5 mg) of the resin was cleaved in 1% TFA/CH_2Cl_2 (0.35 ml) for 30 min. After extensive elution of the peptide with CH_2Cl_2 the combined filtrates were concentrated *in vacuo* and the product was precipitated with ether or recovered by careful removal of the TFA/CH_2Cl_2/ether mixture by rotoevaporation. The crude was dissolved in acetonitrile/water containing 0.1% TFA and subjected to HPLC. Analytical HPLC was performed on a Beckman instrument (System Gold with programmable solvent module 126, diode array detector module 168 and autosampler 507) using a RP C18 column (Vydac) at a flow of 1 ml/min. Solvent A: 5% acetonitrile in water with 0.1% TFA, solvent B: acetonitrile with 0.1% TFA. Peptides were eluted with a linear gradient (0→5 min at 5% B, 5→35% to 95% B, 35→40 min at 95% B, 40→50 min equilibration at 5% A).

VI. Synthesis of Active Ester-glycosylamide Conjugates

Structures of representative active ester-glycosylamide conjugates are shown in Figure 12.

A. Preparation of Ethyleneglycol *mono*(succinimidyl succinate), *mono*(lactosylamidosuccinate)

Ethyleneglycol *bis*(succinimidyl succinate) (335 mg, 0.74 mmol, Pierce) was solubilized in DMF (0.67 mL, heat). After cooling to room temperature, lactosylamine (50 mg, 0.15 mmol) was added. The

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12 Anomeric signals were not resolved due to interference with peptide backbone protons. The extent of defucosylation was assessed from the diagnostic fucose C-6 methyl protons (0.988 ppm, 3x0.48H, d, J = 6.3Hz).

mixture was stirred for 90 min and the reaction was followed by TLC (elution with 1-propanol/ethyl acetate/water, 6:1:3, $R_{f\ educt}$: 0.30, $R_{f\ product}$: 0.58). After complete conversion, product was precipitated with acetone (10 mL). The recovered material was washed with acetone/ether (2:1) and dried *in vacuo*. Yield: 75 mg (0.11 mmol, 73%).

B. Preparation of *Mono*(succinimidyl), *mono*(lactosylamido)suberate

Disuccinimidyl suberate (1 g, 2.71 mmol, Pierce) was dissolved in DMSO (4.32 mL, heat). Lactosylamine (100 mg, 0.29 mmol) was added and the mixture was stirred at 37°C for 90 min. The reaction was followed by TLC (elution with 1-propanol/ethyl acetate/water, 6:1:3, $R_{f\ educt}$: 0.30, $R_{f\ product}$: 0.71)). After complete conversion, ethylacetate (6 mL) was added and product was precipitated with ether (20 mL). Following centrifugation, the residue was washed with ethylacetate/acetone (1:1). Yield 112 mg (66%).

C. Preparation of *Mono*(succinimidyl), *mono*(3'-sialyllactosylamido)suberate

Disuccinimidyl suberate (304 mg, 0.83 mmol, Pierce) and 1-hydroxybenzotriazole (107 mg) was dissolved in DMSO (1.25 mL, heat). After cooling to room temperature, 3'-sialyllactosylamine (50 mg, 79 µmol) was added and the mixture was stirred for 2 hours. The reaction was followed by TLC (elution with 1-propanol/ethyl acetate/water, 6:1:3, $R_{f\ educt}$: 0.40, $R_{f\ product}$: 0.67). After complete conversion, product was precipitated with acetone/ether (1:2, 10 mL). Following centrifugation, the residue was washed with acetone/ether (1:1, 10 mL) and dried *in vacuo*. Yield: 60 mg (86%).

D. Preparation of other Glycosylamide-active ester conjugates

Following the procedures set forth above, the following conjugates were prepared.

PRODUCT	m/z[13]	^{1}H-NMR[14]
Ethyleneglycol *mono*(succinimidyl succinate) *mono*(maltosylamidosuccinate)	682/683.2 ($M+H^+$)	4.894 ppm (d, 8.3 Hz).
Ethyleneglycol *mono*(succinimidyl succinate) *mono*(lactosylamidosuccinate)	N.D.	4.916 ppm (d, 8.6 Hz)
Mono(succinimidyl) *mono*(lactosylamido)suberate	594/595.3 ($M+H^+$)	4.906 ppm (d, 8.6 Hz).
Mono(succinimidyl) *mono*(3'-sialyllactosylamido)suberate	N.D.	4.988 ppm (d, 8.7 Hz).
Mono(succinimidyl) *mono*(lactosyl-N-acetylamido)suberate	635/561.2 (M-Su+Na^+)[15]	5.062 ppm (d, 9.1 Hz)
Mono(succinimidyl) *mono*(chitobiosylamido)suberate	676/602.3 (M-Su+Na+)[15]	5.091 ppm (d,8.9 Hz)
Mono(succinimidyl) *mono*(GlcNAc-6-phosphate-amido)suberate	597/501.1 ($M_{disodium\ salt}$-Su+H^+)[15]	5.091 ppm (d, 8.9 Hz)
Mono(succinimidyl) *mono*(GlcNAc-6-sulfate-amido)suberate	575/501.1 ($M_{sodium\ salt}$-Su+Na^+)[15]	5.091 ppm (d, 8.9 Hz)

13 Calculated/found by low resolution mass spectroscopy.

14 For the N-linked anomeric proton (δ and J).

15 M-Su is the conjugate without the succinimidyl (Su) group. The corresponding free acid is a FAB-MS induced artifact. The intact succinimidyl ester is found in the proton-NMR: 2.878 (4 H, s, N-succinimidyl ester). The free N-hydroxy succinimide resonates at higher field (2.173 ppm, 4 H, s). Also indicative is the shift of the methylene protons of suberic acid: the methylene group neighbouring the glycosylamide linkage resonate at 2.663 ppm (2 H, t, a-CH2) and 1.665 ppm (2 H, t, b-CH2) respectively, the methylene groups next to the succinimidyl ester resonate at 2.264 ppm (2 H, t, a'-CH2) and 1.569 ppm (2 H, t, b'-CH2).

VII. Preparation of Glycoconjugated Beads

A. Bead Preparation

A typical example for a resin-sugar conjugate is given below. Accordingly prepared were maltose-TentaGel and 3'-siallyllactose-TentaGel.

Mono(succinimidyl), *mono*(lactosylamido)suberate (102 mg, 171 mmol) was dissolved in DMF (1 mL) and mixed with TentaGel S NH_2 (156 mg, 0.25 mmol/g, 39 µmol amino groups, Rapp Polymere). After sonication for 1 hour and vortexing for 20 more hours, beads were recovered by centrifugation and washed with DMF and water and lyophilized.

Following the above procedure, conjugates of maltose, siallyllactose, chitobiose, LacNAc, GlcNAc6SO_3^- and GlcNAc6PO_3^{2-} were prepared.

B. Solid Phase Assays

Carbohydrates were detected on beads using adapted digoxigenin immuno assays (Glycan Detection Kit, Glycan Differentiation Kit, Boehringer Mannheim, see product sheets for detailed information)

Ca. 5 mg beads were incubated in the following order:

1) 1 mL periodate solution (14 mmol/L) for 1 hour,

2) 10 µL digoxigenin-hydrazine (5 mmol/L in DMF) plus 0.2 mL acetate buffer for 1 hour,

3) 1 mL casein solution (2% w/v in tris buffered saline (TBS)) for 1 hour,

4) 1 mL anti-digoxigenin-alkaline peroxidase conjugate (1 ng/mL) for 1 hour, and

5) 1 mL nitro-blue tetrazolium staining solution, overnight.

Alternatively, 2.5 mg beads were incubated in the following order:

1) 1 mL casein (2% w/v) in TBS for 1 hour,

2) 0.1 mL digoxigenin-lectin conjugate (RCA 120 at 1 mg/mL, MAA at 0.2 mg/mL) for 1 hour,

3) 1 mL anti-digoxigenin-alkaline peroxidase conjugate (1 ng/mL) for 30 min, and

4) 1 mL of nitro-blue staining solution for 5 min with RCA 120, overnight with MAA.

Figure 4 provides representative lectin stains for beads treated with digoxigenin-labeled MAA (sialic acid specific lectin). Bound lectin was detected by means of an anti-digoxigenin/peroxidase conjugate and subsequent development. From left to right: TentaGel, maltosyl-TentaGel, sialyllactosyl-TentaGel, lactosyl-TentaGel.

C. FACS Analysis

According to an alternative procedure, 2.5 mg 10 µm diameter polystyrene resin beads were incubated in the following order:

1) 0.35 mL Fluorokine cell wash buffer (R&D) for 30 minutes, and

2) 0.3 mL of WGA-fluorescein (Molecular Probes, 0.3 mg/mL) for 30 minutes.

3) Bead suspensions were then subjected to fluorescence activated particle analysis on a FACScan instrument (Becton-Dickinson). For each histogram, 10,000 events were acquired.

VIII. Preparation of Glycosyl Biotinamidocapronamides (Biotinylglycosides)

A. Preparation of Lactosyl Biotinamidocapronamide

As a general example, the preparation of lactosyl biotinamidocapronamide is given:

Lactosylamine (10 mg, 29 µmol) was added to a solution of N-hydroxysuccinimidyl biotinamidocaproate (66 mg, 145 µmol, Pierce) and HOBt (20 mg, 148 µmol) in anhydrous DMSO (0.1 mL, Aldrich). The solution was vortexed for 2 hours. TLC (elution with 1-propanol/ethyl acetate/water, 6:1:3, R_f educt: 0.30, R_f product: 0.65) was used to follow the course of reaction. Product was precipitated with acetone (1.25 mL), recovered by centrifugation and washed with acetone. The material was suspended in methanol (0.15 mL) and reprecipitated with acetone (0.475 mL). After centrifugation this step was repeated. The residue was then washed with ether and dried in vacuo. Yield: 12.8 mg (19 µmol, 66%).

B. Preparation of Other Biotinylglycosides

Following the procedure set forth above, the following biotinylglycosides were prepared:

SACCHARIDE	m/z[16]	m/z[17]	^{1}H-NMR[18]
Glc	N.D.	N.D.	4.954 ppm (d, 9.2 Hz)
Gal	518/541.3 ($M+Na^+$)	N.D.	4.901 ppm (d, 8.0 Hz)
Fuc	502/503.5 ($M+H^+$)	503.2539/503.2555	4.888 ppm (d, 8.8 Hz)
Man	Not isolated		
Xyl	Not isolated		
GlcNAc	559/582.1 ($M+Na^+$)	N.D.	5.072 ppm (d, 9.7 Hz)
GalNAc	559/582.3 ($M+Na^+$)	N.D.	5.014 ppm (d, 9.7 Hz)
ManNac	559/582.3 ($M+Na^+$)	582.2574/582.2569,	5.377 ppm (s) and 4.450 ppm (d, 4.1 Hz)
Neu5Ac	669/670.2 (Msodium salt+H^+)	N.D.	no anomeric proton.
MurNAc	N.D.	N.D.	N.D.
All-2-NAc	559/560.3 ($M+H^+$)	560.2754/560.2756	5.295 ppm (d, 10.1 Hz)
All-3-NAc	Not isolated		
GlcA	Not isolated		
GalA	Not isolated		
GlcNAc-3-sulfate	N.D.	N.D.	5.196 ppm (d, 9.9 Hz)
GlcNAc-6-sulfate	661/684.3 (Msodium salt+Na^+)	684.1961/684.1968	5.099 ppm (d, 9.7 Hz)
GlcNAc-6-phosphate	N.D.	N.D.	N.D.

16 Calculated/found by low resolution mass spectroscopy

17 Calculated/found by high resolution mass spectroscopy.

18 For the N-linked anomeric proton (δ and J).

GlcN-2,3-disulfate	721/744.1 ($M_{disodium\ salt}+Na^+$)	N.D.	N.D.
GlcN-2,6-disulfate	721/744.1 ($M_{disodium\ salt}+Na^+$)	744.1243/744.1225	4.965 ppm (d, 9.3 Hz)
Man-6-phosphate	620/621.1	621.1971/621.1961	5.244 ppm (s)
Gal-6-phosphate	642/643.2 ($M_{disodium\ salt}+H^+$)	643.1791/643.1791	4.938 ppm (d, 8.5 Hz)
Gal-6-sulfate	620/643.2 ($M_{sodium\ salt}+Na^+$)	643.1696/643.1694	4.965 ppm (d, 9.0 Hz)
Glc(α1-4)Glc	680/681.5 ($M+H^+$)	681.3017/681.3032	4.975 ppm (d, 9.3 Hz)
Glc(α1-4)Glc(α1-4)Glc	842/865.3 ($M+Na^+$)	N.D.	4.974 ppm (d, 9.1 Hz)
Glc(β1-4)Glc	680/681.2 ($M+Na^+$)	N.D.	4.516 ppm (d, 7.8 Hz)
Gal(β1-4)Glc	680/681.4 ($M+H^+$)	681.3017/681.3025	5.005 ppm (d, 9.4 Hz)
Neu5Ac(α2-3)Gal(β1-4)Glc	N.D.	N.D.	4.984 ppm (d, 8.9 Hz)
Neu5Ac(α2-6)Gal(β1-4)Glc	993/994.4 ($M_{sodium\ salt}+H^+$)	994.3791/994.3775	4.986 ppm (d, 10.2 Hz)
Neu5Ac(α2-6)Gal(β1-4)GlcNAc	1034/1035.5 ($M_{sodium\ salt}+H^+$)	1035.4056/1035.4058	N.D.
Fuc(α1-2)Gal(β1-4)Glc	N.D.	N.D.	N.D.
Gal(β1-4)Fuc(α1-3)Glc	N.D.	N.D.	N.D.
Gal-2-Me(β1-4)Glc	694/717.2 ($M+Na^+$)	717.2993/717.2991	5.008 ppm (d, 9.3 Hz)

GlcNAc(β1-4)GlcNAc	762/785.2 ($M+Na^+$)	785.3367/785.3374	5.069 ppm (d, 9.4 Hz)
Gal(β1-4)GlcNAc	721/744.3 ($M+Na^+$)	744.3102/744.3087	5.104 ppm (d, 9.1 Hz)
GlcNAc(β1-6)GlcNAc	762/785.2 ($M+Na^+$)	785.3364/785.3374	5.038 ppm (d, 9.7 Hz)
Gal(β1-3)GlcNAc	721/722.3 ($M+H^+$)	722.3282/722.3267	5.123 ppm (d, 9.7 Hz)
Gal(β1-6)GlcNAc	721/722.3 ($M+H^+$)	722.3282/722.3287	5.090 ppm (d, 9.7 Hz)
GlcNAc(β1-6)Gal	721/722.3 ($M+H^+$)	722.3282/722.3276	N.D.
Gal(α1-4)Gal	680/703.2 ($M+Na^+$)	703.2836/703.2850	4.938 ppm (d, 8.8 Hz)
GalA(α1-4)GalA	N.D.	N.D.	4.970 ppm (d, 9.3 Hz)
Gal(β1-6)Gal	680/703.2 ($M+Na^+$)	703.2836/703.2843	4.930 ppm (d, 8.9 Hz)
Gal(β1-3)GalNAc	N.D.	N.D.	5.086 ppm (d, 10.0 Hz)
Gal(β1-4)Man	N.D.	N.D.	N.D.
Gal(β1-3)Ara	N.D.	N.D.	N.D.
Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc	1336/1337.6 ($M_{acid}+H^+$)	1337.5293/1337.5299	N.D.
Neu5Ac(α2-3)Gal(β1-3)(Neu5Ac(α2-6)GalNAc	Not isolated		
Fuc(α1-4)Gal(β1-4)Fuc(α1-3)Glc	972/995.4 ($M+Na^+$)	995.3995/995.3966	4.985 ppm (d, 9.3 Hz)
Gal(β1-3)Fuc(α1-4)GlcNAc(β1-3)Gal(β1-4)Glc	1191/1214.2 ($M+Na^+$)	N.D.	4.992 ppm (d, 9.2 Hz)
Man(α1-3)Man	680/703.2 ($M+Na^+$)	703.2836/703.2840	5.251 ppm (s)

IX. Preparation of Glycoplates

Biotinylglycosides (20 µg/mL in tris buffered saline (TBS, pH 7.5), 0.1 mL/well) were immobilized in streptavidin coated microtiterplates (Pierce) by incubation for 2 hours at room temperature and subsequent washing.

Presence and bioactivity of glycoplates were tested by lectin binding assays:

1) Digoxigenin-labeled lectins (Boehringer Mannheim, 1→10 µL/mL TBS, 0.1 mL/well) were incubated for 1 hour at room temperature and washed off.

2) Anti-digoxigenin alkaline phosphatase conjugate (Boehringer Mannheim, 1 µL/mL TBS, 0.1 mL/well) was incubated for 30 min at room temperature and washed off.

3) Para-nitrophenyl phosphate (Sigma, 5 mg/tablet, 1 tablet/5 mL staining buffer, pH 9.5, 0.1 mL/well) was added and A_{405} was read out in a densitometer. Negative controls were wells without biotinylglycoside. Good signal to background ratios were obtained with the corresponding biotinylglycoside/lectin pairs: LacNAc/DSA, Maltose/Con A, Chitobiose/WGA:, Lac/RCA 120, 3'-sialylLac/MAA, LDFT/AAA, LST c/SNA.

Figure 7 provides ELISA-data for the interaction of streptavidin neoglycoproteins with lectins. Chosen oligosaccharides are reported to bind to the employed lectins. Shorthand notations of the glycosylbiotin/lectin-pairs are given. The background binding (streptavidin without glycosylbiotin) is represented by the right, unlabeled pair of columns respectively.

CONCLUSION

Thus, the advantages of the present invention will now be apparent. Using the methods of the present invention, those skilled in the art will have access to a versatile, efficient method of synthesizing glycoconjugates.

Many other embodiments of the invention will be apparent to those of skill in the art upon reviewing the above description, and to aid in the understanding of the invention, all publications and other references or patent documents in the above description are incorporated herein by reference. The above description is intended to be illustrative and not restrictive, and the scope of the invention should, therefore, be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled.

WHAT IS CLAIMED IS:

1. A method of synthesizing N-linked glycoconjugates, comprising the steps of:

(a) on a surface of a substrate, providing an activated glycosyl acceptor, wherein the activated glycosyl acceptor comprises an activated ester; and

(b) contacting the glycosyl acceptor with a glycosylating agent having a reactive functionality; a coupling catalyst; and an exogenous base,

wherein a substrate-bound glycoconjugate is formed.

2. The method of Claim 1, wherein the step of providing a glycosyl acceptor on a surface of a subrate, further comprises the steps of:

1) on the surface of a substrate, providing a latent glycosyl acceptor bearing a protecting group;

2) removing the protecting group to povide the latent glycosyl acceptor; and

3) activating the latent glycosyl acceptor to provide an activated glycosyl acceptor.

3. The method of Claim 2, wherein the latent glycosyl acceptor comprises a carboxyl group and the step of activating the latent glycosyl acceptor comprises the step of converting the carboxyl group to an -OPfp ester.

4. The method of Claim 1, wherein the solid substrate comprises a surface bearing an oxidized, free carboxyl-containing dextran layer.

5. A method of synthesizing N-linked glycoconjugates, comprising the steps of:

(a) on a surface of a substrate, providing a glycosylating agent having a reactive functionality; and

(b) contacting the glycosylating agent with an activated glycosyl acceptor; a coupling catalyst; and an exogenous base,

wherein a substrate-bound linear glycoconjugate is formed.

6. The method of Claim 5, wherein the activated glycosyl acceptor comprises a compound having a first functional group bearing a first protecting group and an activated carboxyl group.

7. The method of Claim 6, further comprising the steps of:

(c) removing the first protecting group to yield the first functional group; and

(d) repeating the steps of:

(1) coupling a second monomer to the first functional group, the second monomer having a second functional group bearing a second protecting group; and

(2) removing the second protecting group,

until the substrate-bound linear glycoconjugate is formed.

8. The method of Claim 1, 5, or 7, wherein the activated ester comprises an phentafluorophenyl ester.

9. The method of Claim 1, 5, or 7, wherein the glycosylating agent comprises a glycosylamine and the reactive functionality comprises a primary amino group.

10. The method of Claim 1, 5, or 7, further comprising the steps of:

(c) cleaving the glycoconjugate from the surface of the substrate.

11. The method of Claim 10, wherein the glycoconjugate is cleaved from the surface by treatment with 50% trifluoroacetic acid.

12. The method of Claim 5, or 7, wherein the step of providing a glycosylating agent on a surface of the substrate, further comprises the steps of:

1) on the surface of a substrate, providing a primary amino group;

2) coupling a glycuronic acid or glyconolactone to the amino group to yield a substrate-bound sugar having a

hydroxyl group at its reducing end; and

3) converting the hydroxyl group to an amino group.

13. The method of Claim 12, wherein the step of converting the hydroxyl group to an amino group comprises the step of:

treating the substrate-bound sugar having a hydroxyl group at its reducing end with ammonium carbonate in a polar solvent.

14. A method for synthesizing immobilized N-linked glycoconjugates, comprising the steps of:

(a) on a surface of a substrate, providing a reactive functionality; and

(b) contacting an active ester-glycosylamide conjugate with the reactive functionality;

wherein an immobilized N-linked glycoconjugate is formed.

15. The method of Claim 14, wherein the active ester-glycosylamide conjugate is prepared by treating a glycosylamine with an active ester having two activated carboxyl groups.

16. The method of Claim 15, wherein the active ester having two activated carboxyl group is selected from the group consisting of ethyleneglycol *bis*(succinimidyl succinate) and disuccinimidyl suberate.

17. The method of Claim 14, wherein the step of contacting an active ester-glycosylamide conjugate with the substrate-bound reactive functionality comprises the step of contacting the active ester-glycosylamide conjugate with the substrate-bound reactive functionality in the presence of a coupling catalyst.

18. The method of Claim 17, wherein the coupling catalyst comprises HOBt.

19. The method of Claim 14, wherein the solid support comprises a bead.

20. A method for synthesizing immobilized N-linked glycoconjugates, comprising the steps of:

(a) on a surface of a substrate, providing streptavidin in a predefined region;

(b) contacting a glycosylating agent having a reactive functionality; a glycosyl acceptor, wherein the glycosyl acceptor comprises an activated ester of a biotin-derivative; a coupling catalyst; and an exogenous base, to form a biotinylated glycoconjugate; and

(c) contacting the biotinylated glycoconjugate with the substrate-bound streptavidin,

wherein an immobilized N-linked glycoconjugate is formed.

21. The method of Claim 20, wherein the activated ester of a biotin-derivative comprises biotinamidocapronamide.

22. The method of Claim 21, wherein the activated ester comprises an OSu-ester.

23. The method of Claim 20, wherein the substrate comprises a support having a plurality of wells.

24. The method of Claim 23, wherein the support having a plurality of wells is a 96-well microtiter plate.

25. An array of immobilized, N-linked glycoconjugates, the array prepared by:

(a) on a surface of a substrate, providing streptavidin in a predefined region;

(b) repeating the steps of:

1) contacting a glycosylating agent having a reactive functionality; a glycosyl acceptor, wherein the glycosyl acceptor comprises an activated ester of a biotin-derivative; a coupling catalyst; and an exogenous base, to form a biotinylated glycoconjugate,

2) contacting the biotinylated glycoconjugate with the substrate-bound streptavidin in a predefined region ,

until the desired array of immobilized, N-linked glycoconjugates is formed.

26. The array of Claim 25, wherein the activated ester of a biotin-derivative comprises biotinamidocapronamide.

27. The array of Claim 26, wherein the activated ester comprises an OSu-ester.

28. The array of Claim 25, wherein the substrate comprises a support having a plurality of wells.

29. The array of Claim 28, wherein the support having a plurality of wells is a 96-well microtiter plate.

30 A library of N-linked glycoconjugates comprising a plurality of different compounds, each compound covalently linked to a solid support, wherein each of said compounds comprises at least one glycoconjugate linkage which linkage is prepared by the method which comprises the steps of:

(a) on a surface of a substrate, providing an activated glycosyl acceptor, wherein the activated glycosyl acceptor comprises an activated ester; and

(b) contacting the glycosyl acceptor with a glycosylating agent having a reactive functionality; a coupling catalyst; and an exogenous base, under conditions effective to couple the glycosylating agent to the glycosyl acceptor to form a substrate-bound glycoconjugate.

31. The library of Claim 30, wherein at least two different glycosyl acceptors are used.

32. The library of Claim 30, wherein at least two different glycosylating agents are used.

33. The library of Claim 30, wherein at least two different coupling catalysts are used.

34. The library of Claim 30, wherein at least two different exogenous bases are used.

35. The library of Claim 30, wherein at least two different sets of conditions effective to form a substrate-bound glycoconjugate are used.

36. The library of Claim 30, wherein each compound of said plurality of different compounds is covalently linked to the same solid support.

37. The library of Claim 30, wherein the solid support is a 96-well microtiter plate.

38. The library of Claim 30, wherein each compound of said plurality of different compounds is covalently linked to a different solid support.

39. The library of Claim 38, wherein the solid support is a bead.

40. The library of Claim 38, wherein each of the beads further comprises a support-bound identifier tag, wherein the tag identifies a molecule attached thereto.

41. The library of Claim 40, wherein the tag is an oligonucleotide.

42. A library of N-linked glycoconjugates comprising a plurality of different compounds, each compound covalently linked to a solid support, wherein each of said compounds comprises at least one glycoconjugate linkage which linkage is prepared by the method which comprises the steps of:

(a) on a surface of a substrate, providing a glycosylating agent having a reactive functionality; and

(b) contacting the glycosylating agent with an activated glycosyl acceptor; a coupling catalyst; and an exogenous base,

under conditions effective to couple the glycosylating agent to the glycosyl acceptor to form a substrate-bound glycoconjugate.

43. The library of Claim 43, wherein at least two different glycosyl acceptors are used.

44. The library of Claim 43, wherein at least two different glycosylating agents are used.

45. The library of Claim 43, wherein at least two different coupling catalysts are used.

46. The library of Claim 43, whereinat least two different exogenous bases are used.

47. The library of Claim 43, whereinat least two different sets of conditions effective to form a substrate-bound glycoconjugate are used.

48. The library of Claim 43, wherein each compound of said plurality of different compounds is covalently linked to the same solid support.

49. The library of Claim 48, wherein the solid support is a 96-well microtiter plate.

50. The library of Claim 43, wherein each compound of said plurality of different compounds is covalently linked to a different solid support.

51. The library of Claim 50, wherein the solid support is a bead.

52. The library of Claim 51, wherein each of the beads further comprises a support-bound identifier tag, wherein the tag identifies a molecule attached thereto.

53. The library of Claim 52, wherein the tag is an oligonucleotide.

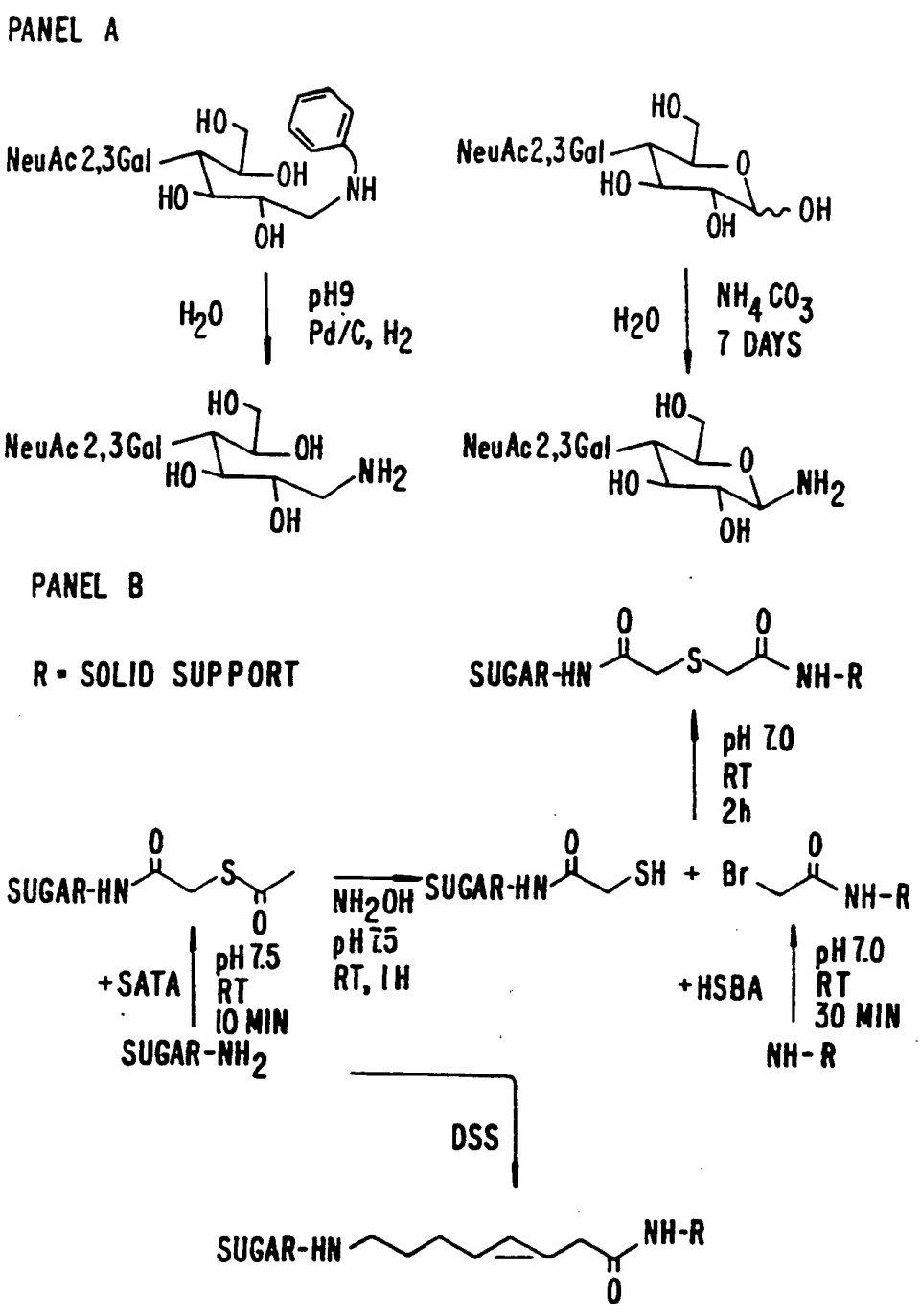

PANEL A
NeuAc 2,3Gal
HO
OH
NH
HO
OH
H_2O
pH9
Pd/C, H_2
NeuAc 2,3Gal
HO
OH
NH_2
HO
OH
NeuAc2,3Gal
O
HO
OH
OH
H_2O
NH_4CO_3
7 DAYS
NeuAc 2,3Gal
HO
OH
NH_2
HO
OH
PANEL B
R = SOLID SUPPORT
SUGAR-HN
O
S
O
NH-R
pH 7.0
RT
2h
SUGAR-HN
O
S
O
+SATA
pH 7.5
RT
10 MIN
SUGAR-NH_2
NH_2OH
pH 7.5
RT, 1H
SUGAR-HN
O
SH
+
Br
O
NH-R
+HSBA
pH 7.0
RT
30 MIN
NH-R
DSS
SUGAR-HN
NH-R
O

FIG. 1.

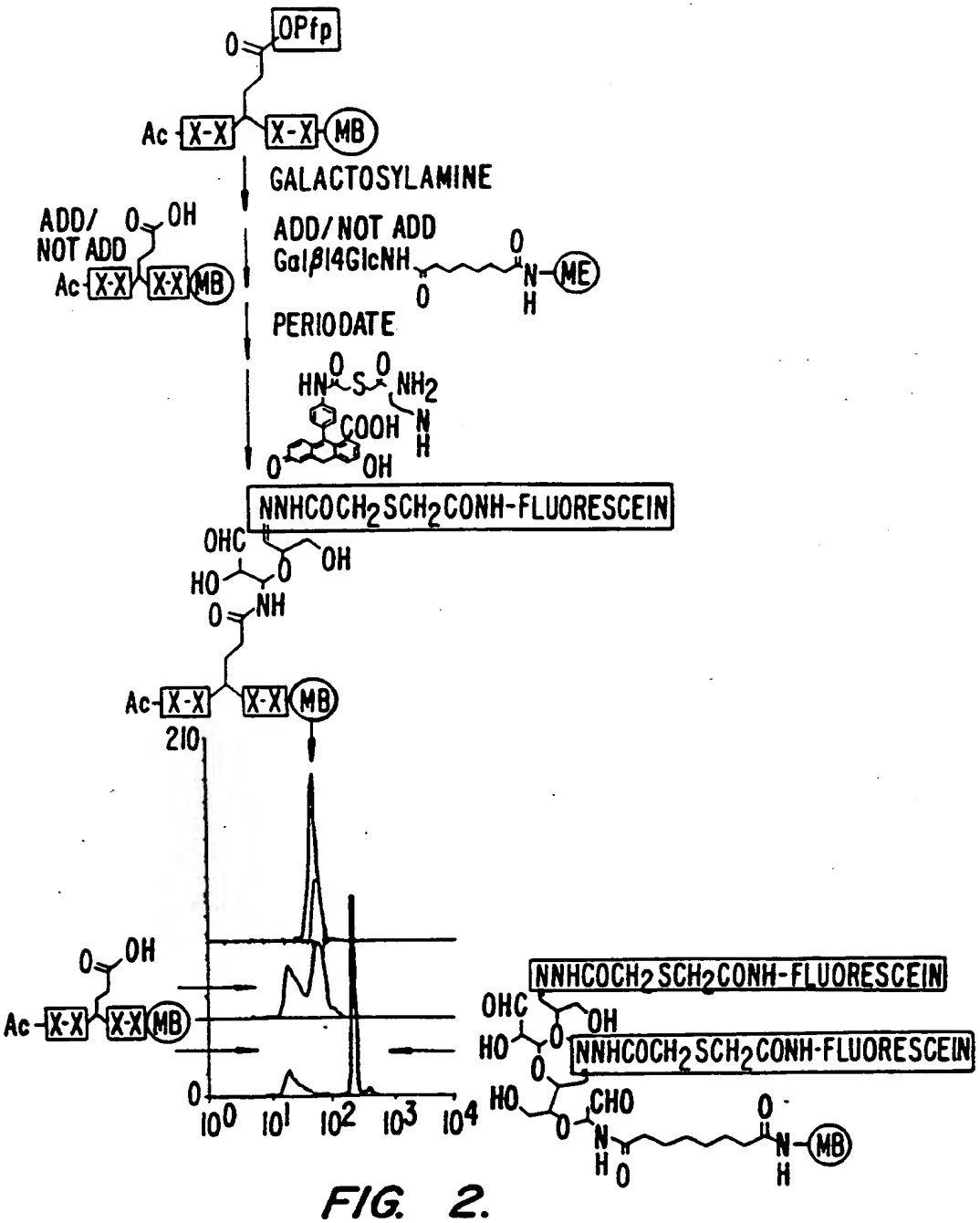

OPfp
Ac X-X X-X MB
GALACTOSYLAMINE
ADD/ NOT ADD
Ac X-X X-X MB
ADD/NOT ADD
Galβ14GlcNH
ME
PERIODATE
NNHCOCH2SCH2CONH-FLUORESCEIN
Ac X-X X-X MB
210
Ac X-X X-X MB
NNHCOCH2SCH2CONH-FLUORESCEIN
NNHCOCH2SCH2CONH-FLUORESCEIN
MB
10^0 10^1 10^2 10^3 10^4

FIG. 2.

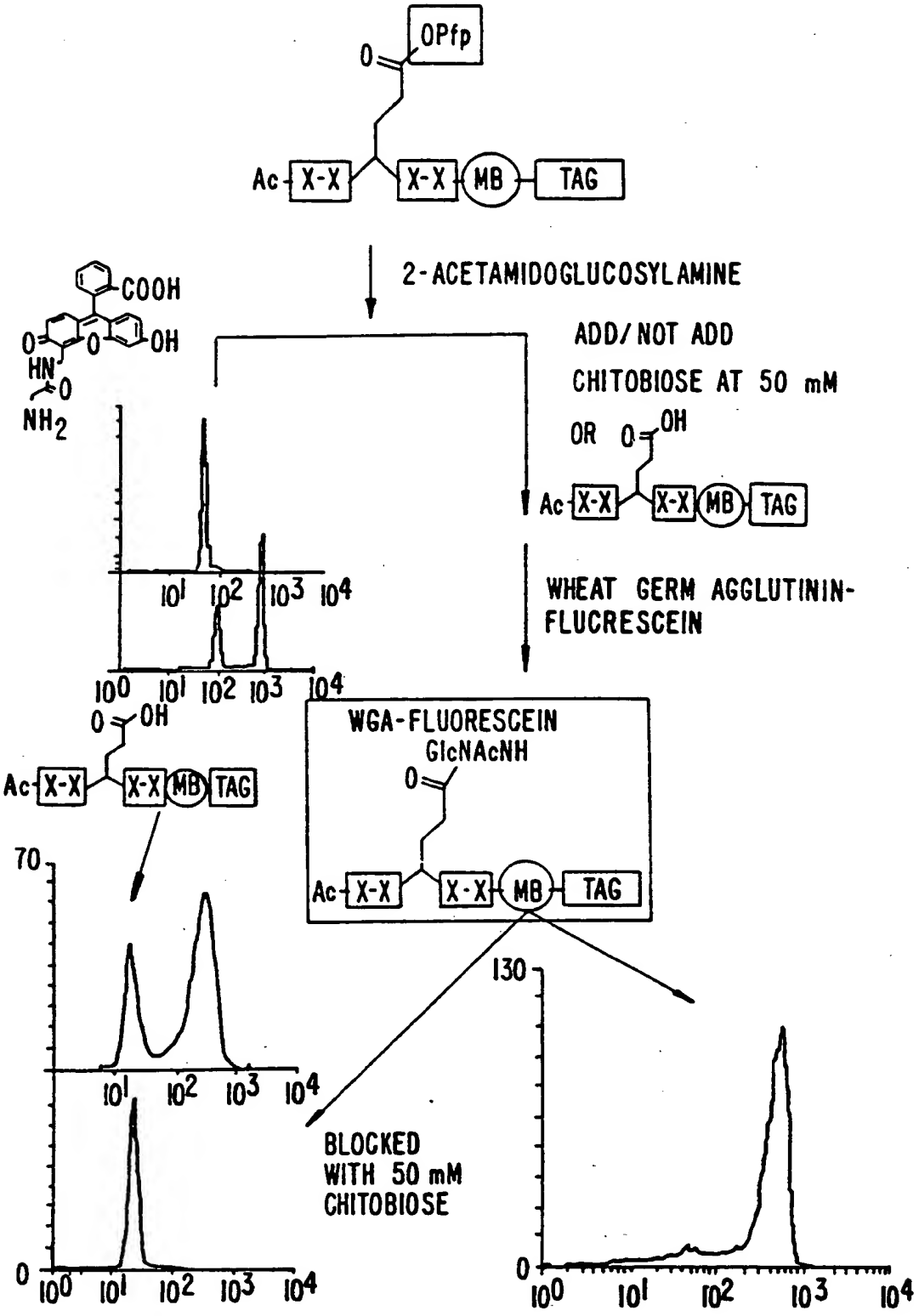

OPfp
Ac X-X X-X MB TAG
2-ACETAMIDOGLUCOSYLAMINE
ADD/NOT ADD
CHITOBIOSE AT 50 mM
OR
WHEAT GERM AGGLUTININ-
FLUCRESCEIN
WGA-FLUORESCEIN
GlcNAcNH
BLOCKED
WITH 50 mM
CHITOBIOSE

FIG. 3.

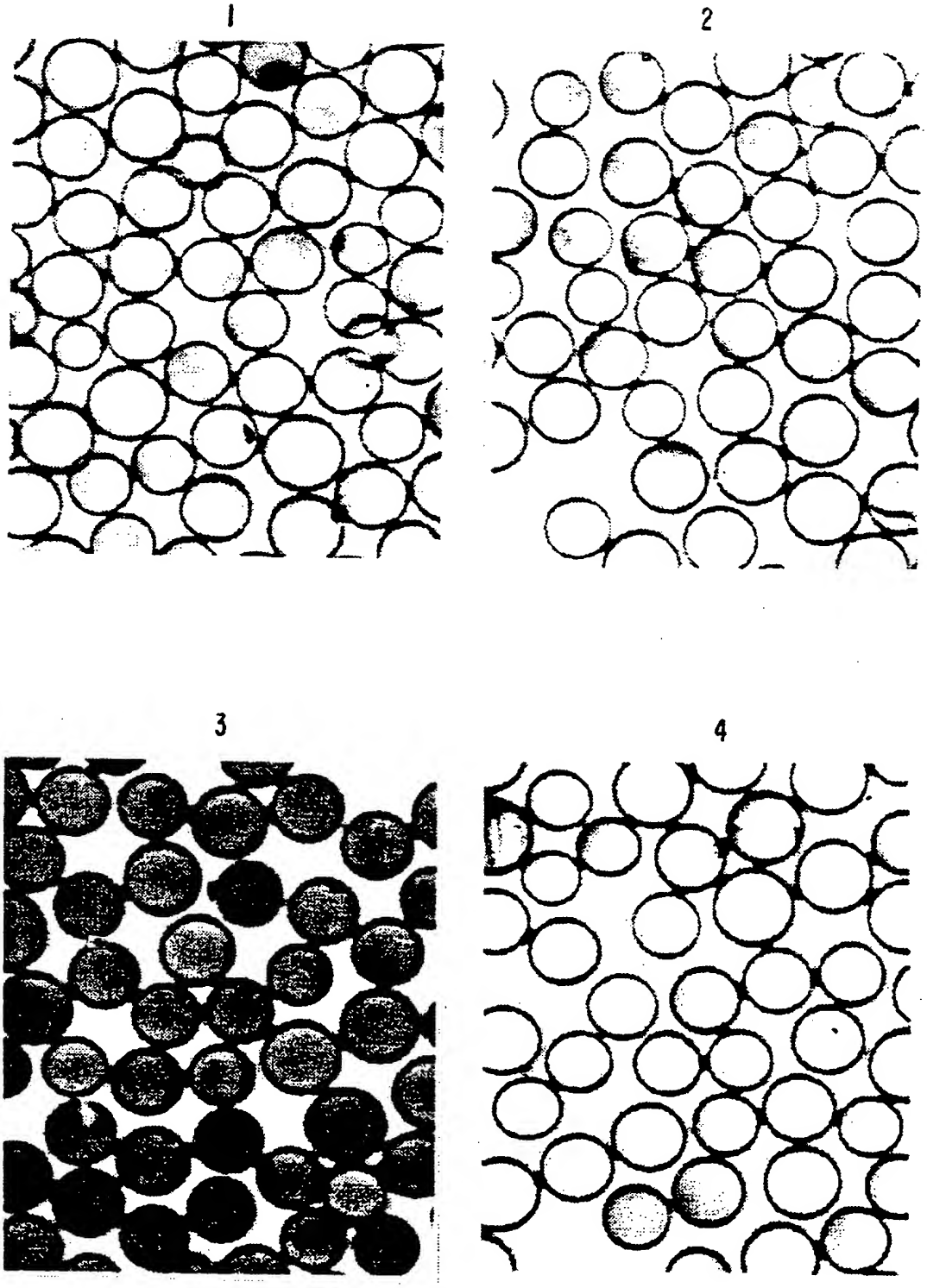


FIG. 4.

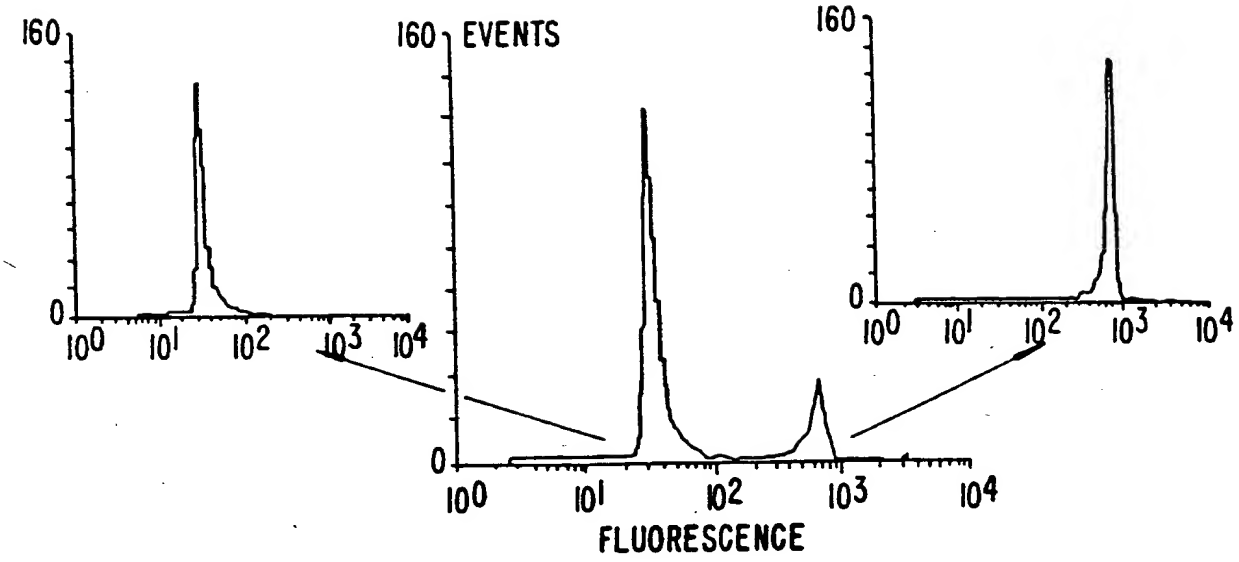
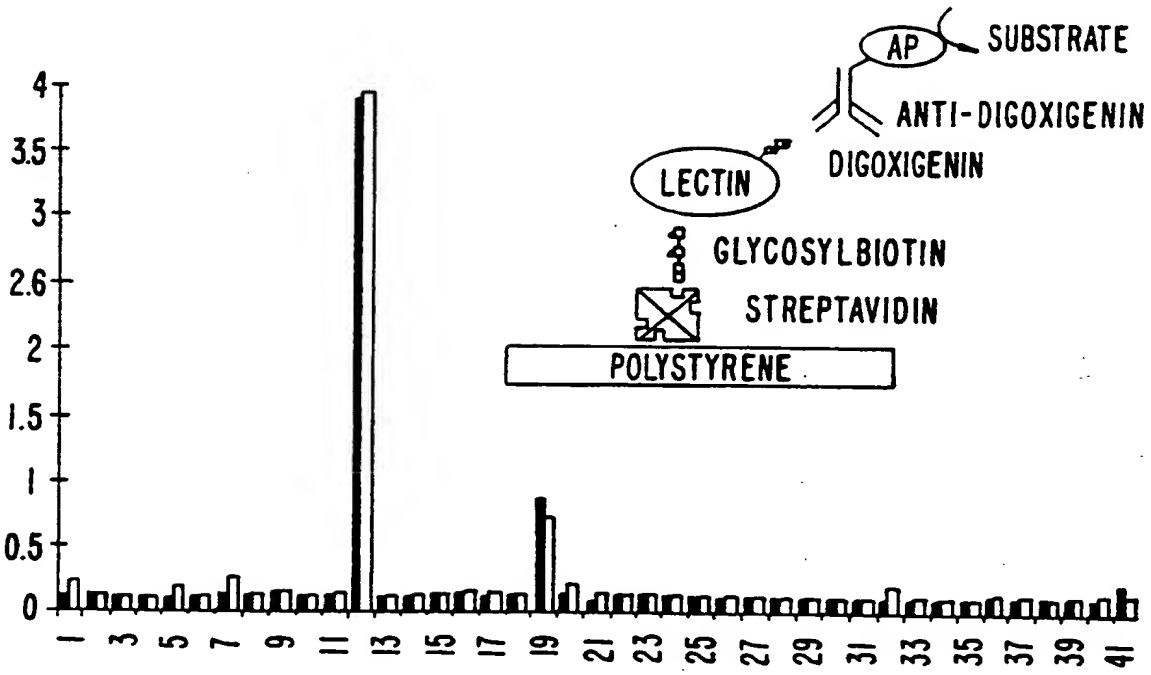

160
0
10^0 10^1 10^2 10^3 10^4
160
EVENTS
0
10^0 10^1 10^2 10^3 10^4
FLUORESCENCE
160
0
10^0 10^1 10^2 10^3 10^4

FIG. 5.



1: Lac, 2: 3'-SLac, 3: Glc, 4: Gal, 5: Fuc, 6: Malt, 7: GlcNAc, 8: GlcNAc-6-SULFATE, 9: GlcNAc-6-PHOSPHATE, 10: MALTOTRIOSE, 11: LacNAc, 12: CHITO, 13: GalNAc, 14: LNFPII, 15: Fuc_2Lac, 16: Gal-6-PHOSPHATE, 17: Galβ6Gal, 18: Galβ6GlcNAc, 19: GlcNAcβ6Gal, 20: Galβ3GlcNac, 21: Galβ3GalNAc, 22: 3'-FucLac, 23: 2-FucLac, 24: GalAα4GalA, 25: Galβ4Man, 26: MurNAc, 27: Galβ3Ara, 28: Manα3Man, 29: Galα4Gal, 30: GlcNAcβ6GlcNAc, 31: 2'-Me-Lac, 32: Man-6-PHOSPHATE, 33: AllNAc, 34: GlcN2,3-DISULFATE, 35: GlcN2, 6-DISULFATE, 36: Gal-6-SULFATE, 37: 6'-SLac, 38: 6'-SLacNac, 39: ManNAc, 40: 6'-SLacNAcLac, 41: Neu5Ac, 42: GlcNAc-3-SULFATE, 43: CELLO

FIG. 6.

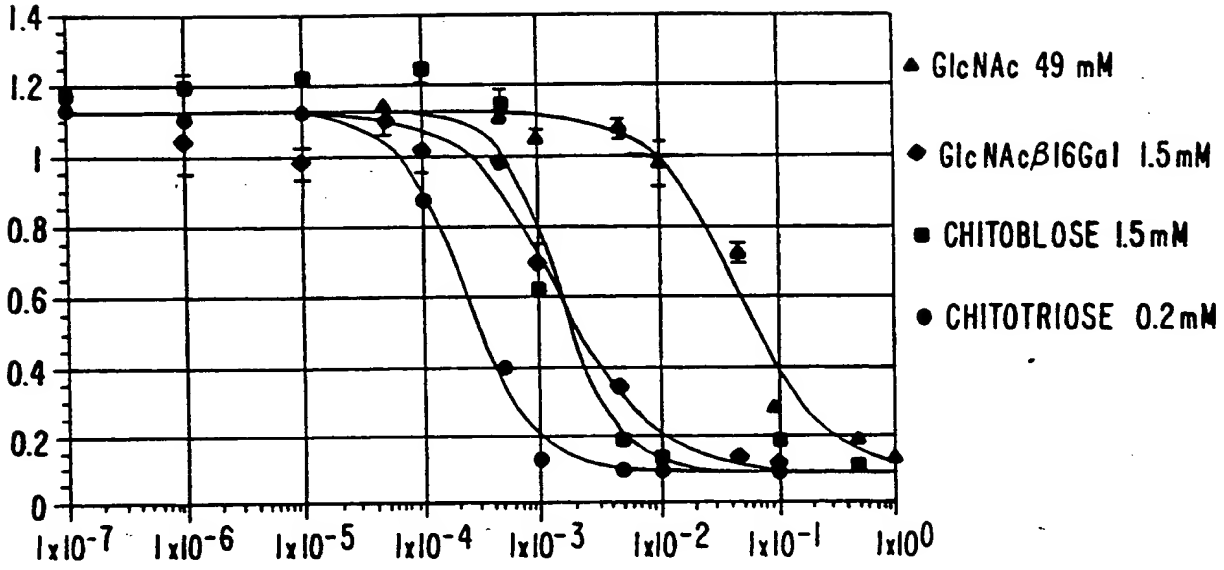
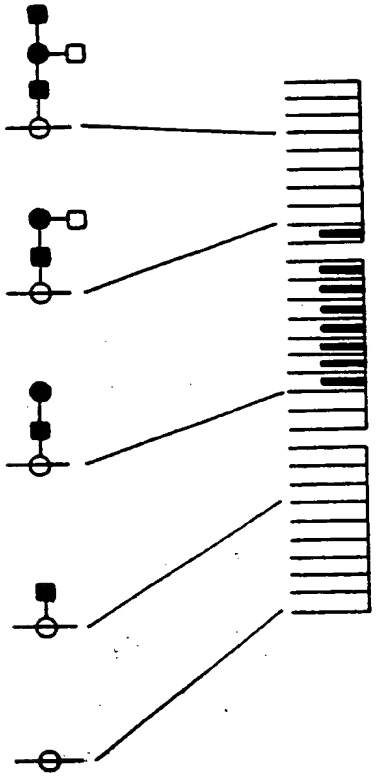

1.4
1.2
1
0.8
0.6
0.4
0.2
0
1x10-7
1x10-6
1x10-5
1x10-4
1x10-3
1x10-2
1x10-1
1x100
▲ GlcNAc 49 mM
◆ GlcNAcβ16Gal 1.5mM
■ CHITOBLOSE 1.5mM
● CHITOTRIOSE 0.2mM

FIG. 7.



1. ALPHA-N-ACETYLGALACTOSAMINIDASE
2. BETA-N-ACETYLGALACTOSAMINIDASE
3. ALPHA-GALACOSIDASE
4. BETA-GALACTOSIDASE
5. ALPHA-MANNOSIDASE
6. BETA-MANNOSIDASE
7. SIALIDASE
8. BETA-N-ACETYLGLUCOSAMINIDASE
9. ALPHA-FUCOSIDASE

STAINS WITH WGA-LECTIN (GlcNAc-SPECIFIC)

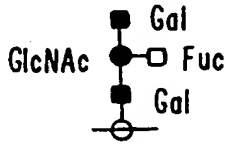


FIG. 8.

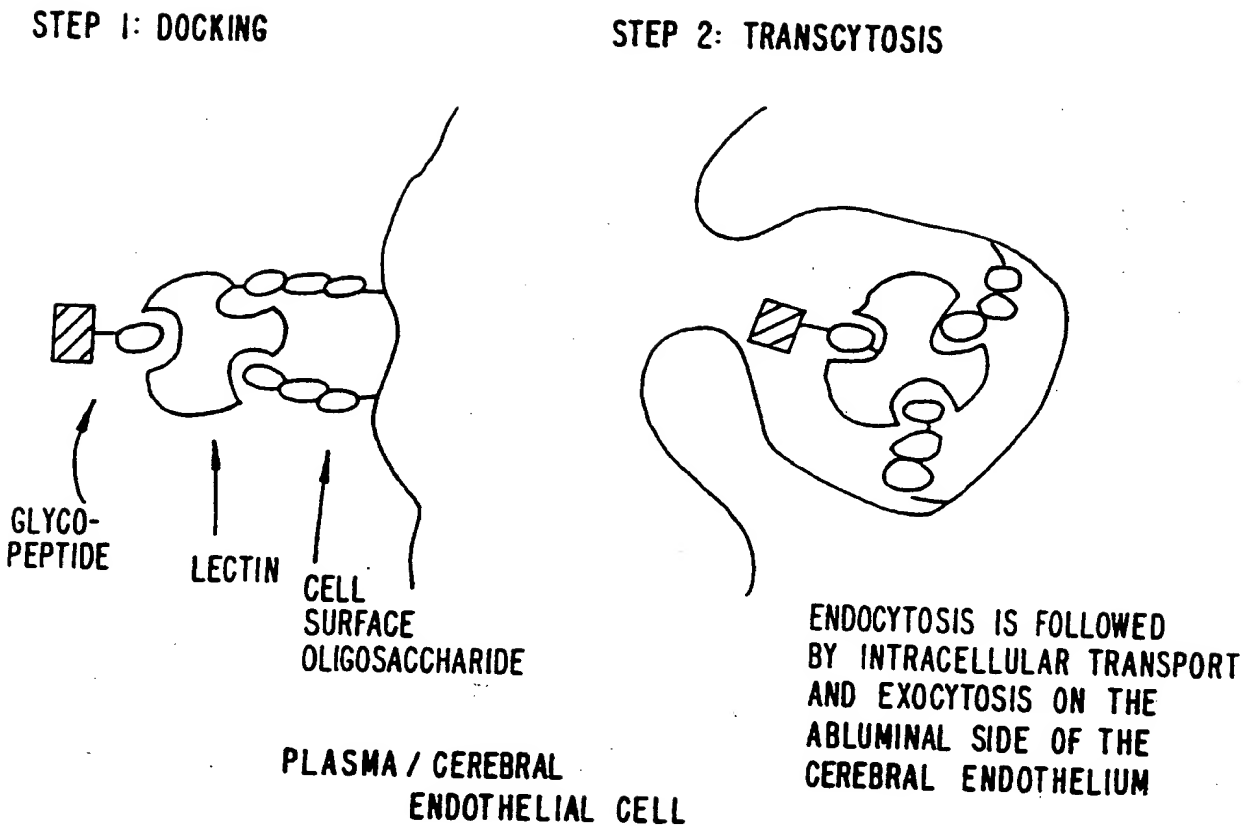

STEP 1: DOCKING
STEP 2: TRANSCYTOSIS
GLYCO-
PEPTIDE
LECTIN
CELL
SURFACE
OLIGOSACCHARIDE
PLASMA / CEREBRAL
ENDOTHELIAL CELL
ENDOCYTOSIS IS FOLLOWED
BY INTRACELLULAR TRANSPORT
AND EXOCYTOSIS ON THE
ABLUMINAL SIDE OF THE
CEREBRAL ENDOTHELIUM

FIG. 9.

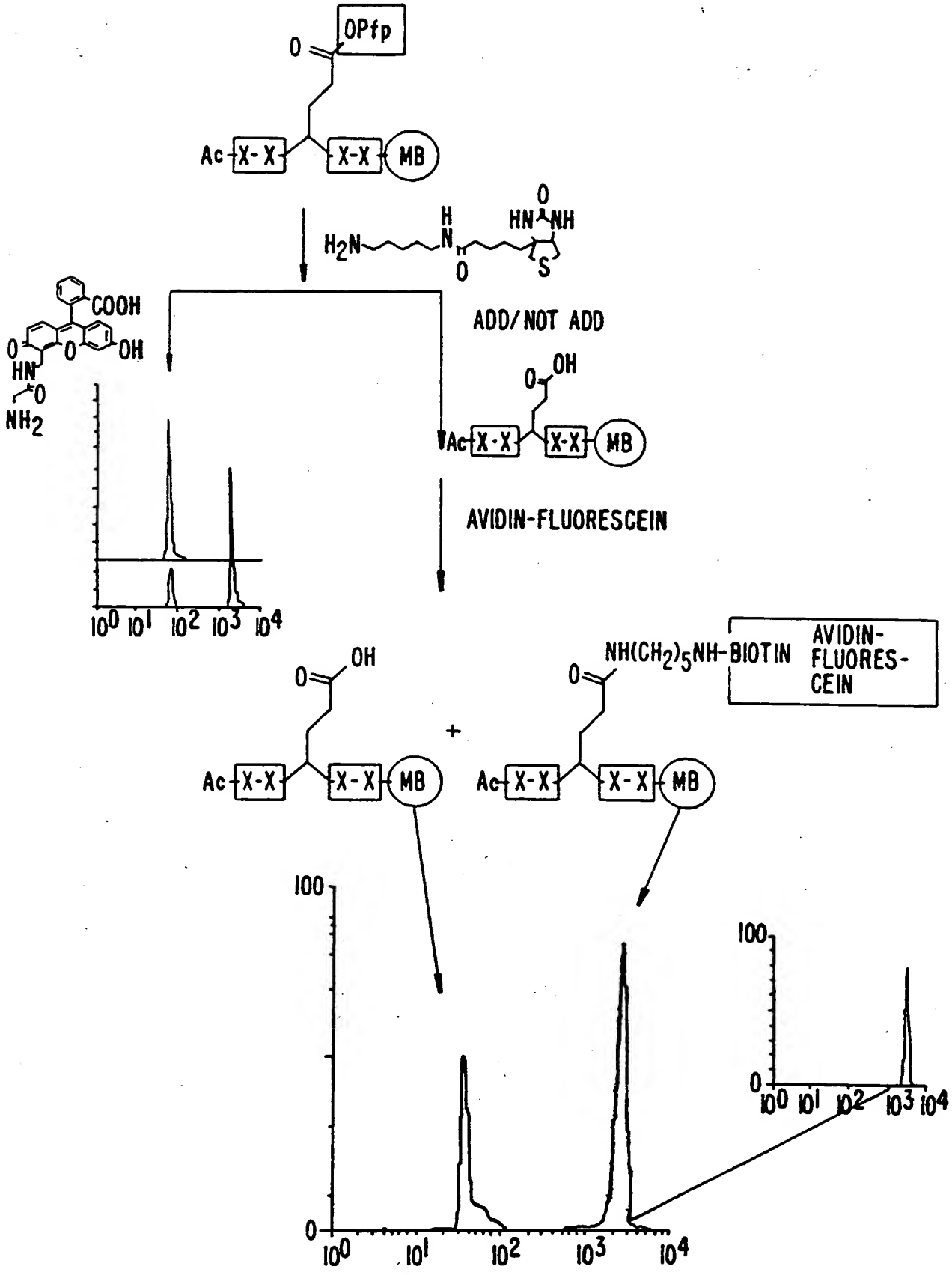

OPfp
Ac X-X X-X MB
H2N
HN NH
ADD/NOT ADD
COOH
OH
NH2
AVIDIN-FLUORESCEIN
NH(CH2)5NH-BIOTIN
AVIDIN-FLUORES-CEIN
100
0
10^0 10^1 10^2 10^3 10^4

FIG. 10.

[Fmoc]-E[OAll]Y[tBu]GGFL-Sasrin (1)

$Pd(PPh_3)$ IN NMM/HAc/$CHCl_3$

[Fmoc]-E[OH]Y[tBu]GGFL-Sasrin (2)

+ $CF_3COOPfp$ IN Pyr/DMF

[Fmoc]-E[OPfp]Y[tBu]GGFL-Sasrin (3)

+ $GalNH_2$, DIEA, HOBt IN DMSO

[Fmoc]-Q[Galβ]Y[tBu]GGFL-Sasrin (4)

Pip/DMF

TFA/CH_2Cl_2

[H]-Q[Galβ]YGGFL-[OH] (5)

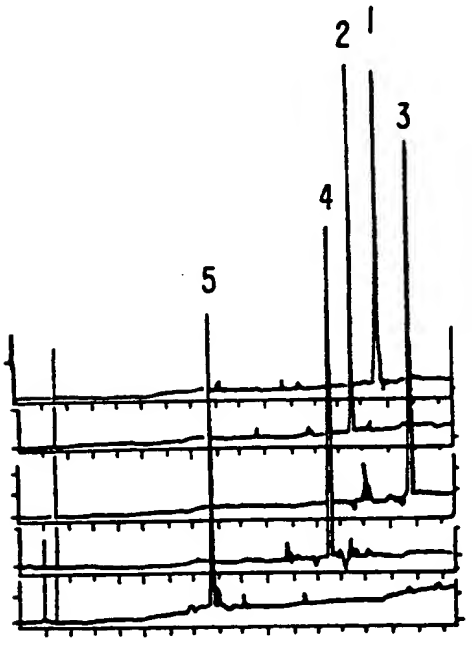


FIG. 11.

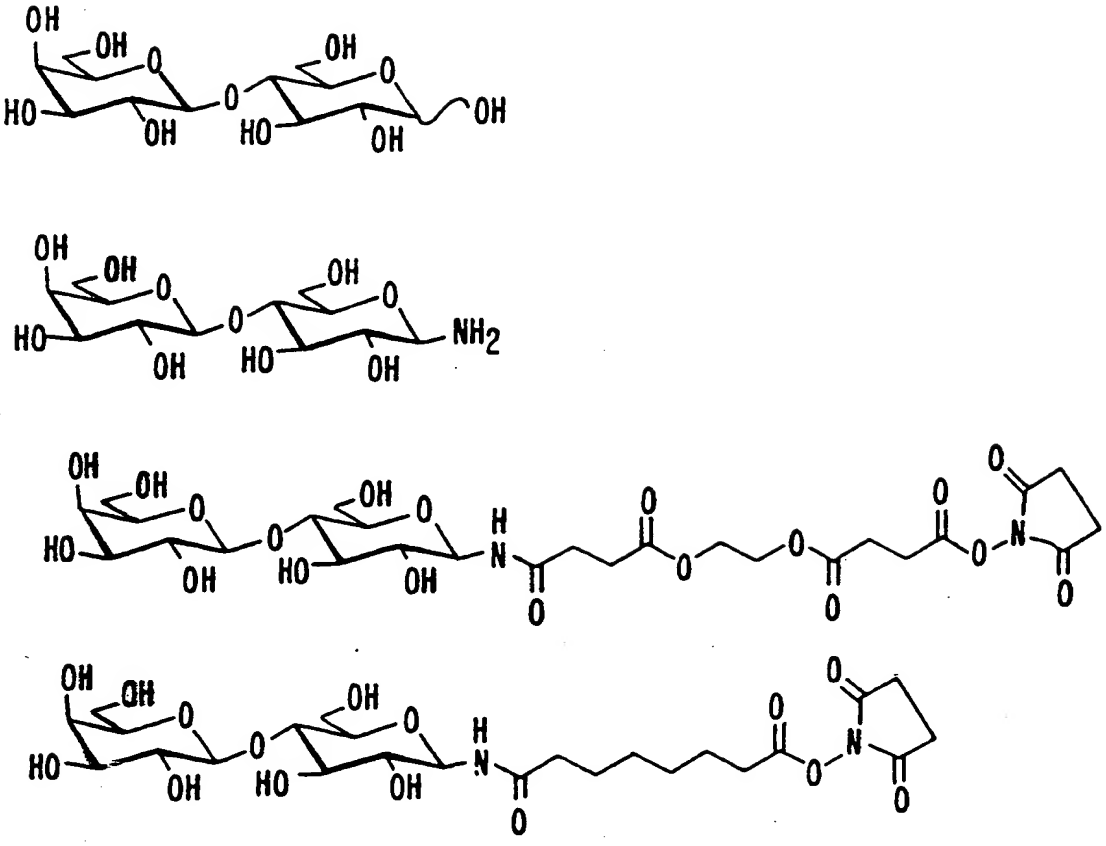


FIG. 12.

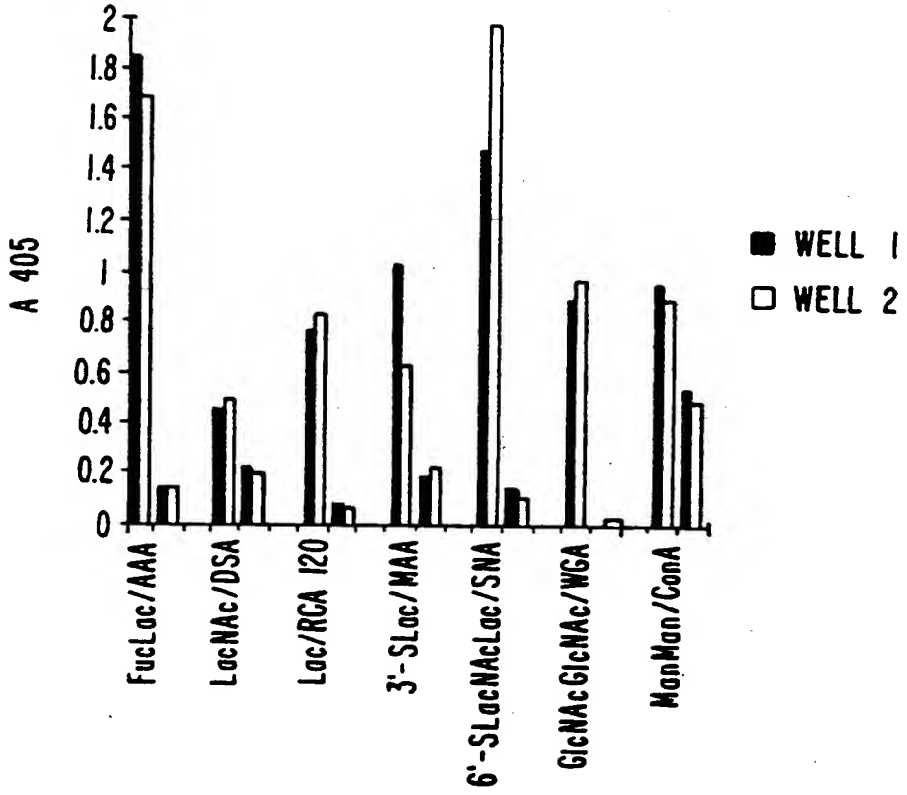

A 405
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
FucLac/AAA
LacNAc/DSA
Lac/RCA 120
3'-SLac/MAA
6'-SLacNAcLac/SNA
GlcNAcGlcNAc/WGA
ManMan/ConA
WELL 1
WELL 2

FIG. 13.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00484

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/543; C07K 1/04; C07H 1/00
US CL :436/518; 530/345; 536/55.3
According to International Patent Classification (IPC) or to both nati nal classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system f llowed by classification symbols)

U.S. : 436/518, 528, 531, 532, 533, 534; 530/345, 333, 334; 536/55.3, 53, 55.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS
search terms: gycopeptides, glycosylated peptides, synthesis, N-linked glycoconjugates

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TETRAHEDRON LETTERS, VOLUME 32, NUMBER 29, ISSUED 1991, URGE ET AL., "SOLID-PHASE SYNTHESIS OF GLYCOPEPTIDES: SYNTHESIS OF N(ALPHA)-FLUORENYLMETHOXYCARBONYLL-ASPARAGINEN(BETA)-GLYCOSIDES", PAGES 3445-3448, SEE ENTIRE DOCUMENT.	1, 5-13, 30-39, 40, 41
Y	SCHNEIDER ET AL., "PEPTIDES 1992", PUBLISHED 1993 BY ESCOM SCIENCE PUBLISHERS B.V., PAGES 191-193, SEE PAGE 192.	1-4, 8-11, 30-41
Y	ACCOUNTS OF CHEMICAL RESEARCH, VOLUME 11, ISSUED 1978, LEZNOFF, "THE USE OF INSOLUBLE POLYMER SUPPORTS IN GENERAL ORGANIC SYNTHESIS", PAGES 227-233, SEE PAGE 227.	1-13, 30-41

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date f the actual completion of the international search	Date of mailing f the international search report
03 MARCH 1995	06 APR 1995

Name and mailing address of the ISA/US	Authorized fficer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Lora M. Green Deborah Frieman for
Facsimile No. (703) 305-3230	Telephone N . (703) 308-0196

F rm PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US95/00484

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Categ ry*	Citation f document, with indicati n, where appropriate, of th relevant passages	Relevant to claim No.
Y	BIOCHEMISTRY, VOLUME 31, NUMBER 44, ISSUED 1992, MANGER ET AL., "SYNTHESIS OF 1-N-GLYCYL B-OLIGOSACCHARIDE DERIVATIVES. REACTIVITY OF LENS CULINARIS LECTIN WITH A FLUORESCENT LABELED STREPAVIDIN PSEUDOGLYCOPROTEIN AND IMMOBILIZED NEOGLYCOLIPID", PAGES 10733-10740, SEE FIGURE 4.	14-16, 17-29, 42-63
Y	US, A, 5,166,315 (SUMMERTON ET AL.) 24 NOVEMBER 1992, COLUMN 25, LINES 61-68.	14-16, 17-19
Y	VIGNANA BHARATHI, VOLUME 2, NUMBER 1, ISSUED 1977, KHAN ET AL., "SOLID-PHASE PEPTIDE SYNTHESIS: ACCELERATING INFLUENCE OF 1-HYDROXYBENZOTRIAZOLE ON THE COUPLING RATES", PAGES 1-4, SEE ABSTRACT.	17-29, 42-53
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